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TESIS DOCTORAL

Especie, filogeografía y producción de extrolitos en *Bryoria y Pseudephebe (Parmeliaceae)*

Species, philogeography and extrolite production in *Bryoria and Pseudephebe (Parmeliaceae)*

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Carlos Galán Boluda

Directores

David Leslie Hawksworth
Víctor Jiménez Rico

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Carlos Galán Boluda

Memoria de tesis doctoral

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Estudio realizado bajo la dirección de los doctores:

David Leslie Hawksworth y

Víctor Jímenez Rico

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El Dr. David L. Hawksworth, professor Contratado Doctor en la Universidad Complutense de Madrid, y el Dr. Víctor Jiménez Rico, profesor Titular de Universidad en la Universidad Complutense de Madrid,

CERTIFICAN,

que el Graduado Carlos Galán Boluda ha realizado, bajo nuestra dirección, la tesis doctoral titulada "**Especie, filogeografía y producción de extrolitos en *Bryoria* y *Pseudephebe* (*Parmeliaceae*)** ", y que cumple con todos los requisitos necesarios para optar al grado de doctor por la Universidad Complutense de Madrid.

Fdo.: David L. Hawksworth
Vº Bº Director de la tesis

Fdo.: Víctor Jiménez Rico
Vº Bº Director de la tesis

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Índice

Nomenclatura, autorías y abreviaturas

Las abreviaturas de los herbarios y autores pueden consultarse respectivamente en: http://sweetgum.nybg.org/ih/ y http://www.indexfungorum.org/Names/AuthorsofFungalNames.asp

Las autorías de los taxones que se mencionan se corresponden con las que aparecen en las bases de datos nomenclaturales Index Fungorum (http://www.indexfungorum.org) y Mycobank (http://www.mycobank.org).

El siguiente listado muestra las principales abreviaturas utilizadas a lo largo de esta memoria doctoral. No incluye abreviaturas de uso generalizado y estandarizadas.

Listado de abreviaturas:

- A: disolvente A usado en TLC, tolueno/dioxano/ácido acético (180:45:5).
- ABGD: Automatic Barcode Gap Discovery.
- *ad int.*: *ad interim*, hasta el moment, provisionalmente.
- *aff.*: *affinis*, afín, parecido a, relacionado con, limitando con.
- *agg.*: *aggregatum*, agregado, grupo de especies relacionadas.
- AMOVA: análisis de varianza molecular.
- ANOVA: análisis de la varianza.
- B: disolvente B usado en TLC, dioxano/metil-butil eter/ácido fórmico (140:72:18).
- bp: base pairs, pares de bases.
- BP&P: Bayesian Phylogenetics and Phylogeography.
- C: disolución de hipoclorito de sodio usada como reactivo.
- C: disolvente C en TLC, tolueno/ácido acético (170:30).
- *c.*: *circa*, cerca de, alrededor de.
- *cf.*: *confer*, a confirmar, a cotejar.
- DIC: Differential Interference Contrast, microscopía por contraste de fases.
- *e.g.*: *exempli gratia*, por ejemplo.
- *et al.*: *et alii*, *aliorum*, y otro, y otros.
- *Fig.*: Figura.
- G: disolvente G en TLC, tolueno/etil-acetato/ácido fórmico (139:83:8).
- HPLC: High Performance Liquid Chromatography, cromatografía líquida de alta eficacia.
- *Ibid.*: *ibidem*, lo mismo, en el mismo lugar.
- ITS: Internal Transcribed Spacer, espaciador interno transcrito.

- K: disolución concentrada de hidróxido de potasio usada como reactivo.
- LD: Linkage Desequilibrium, desequilibrio de ligamiento.
- LSU: Large Ribosomal Subunit, subunidad grande del ADN ribosómico.
- *MCM7*: Minichromosome Complex Maintenance component 7.
- min: minuto.
- ml: mililitro.
- ML: Maximum Likelihood, máxima verosimilitud.
- Mt: monte.
- mt: mitocondrial.
- Mya: Million years ago, millones de años.
- ng: nanogramo.
- nm: nanómetro.
- No.: número.
- nu: nuclear.
- PCR: Polymerase Chain Reaction, reacción en cadena de la polimerasa.
- Pd: parafenilendiamina en solución alcohólica usada como reactivo.
- PKS: Polyketide Synthase, policétido sintasa.
- PTP: Poison Tree Processes.
- *sect.*: *sectio*, sección.
- SEM: Scanning Electron Microscope, microscopio electrónico de barrido.
- *s. l.*: *sensu lato*, en sentido amplio.
- *sp. nov.*: especie nueva.
- s. s.: sustituciones por sitio.
- *s. str.*: *sensu stricto*, en sentido estricto.
- SSU: Small Ribosomal Subunit, subunidad pequeña del ADN ribosómico.
- *RPB*1: ADN codificante para la subunidad mayor de la ARN polimerasa II.
- TLC: Thin Layer Chromatography, cromatografía en capa fina.
- UV: luz ultravioleta.
- v: versión.
- *viz.: videlicet*, a saber, es decir.
- yr: year, año.
- µl: microlitro.

Resumen

Título

Especie, filogeografía y producción de extrolitos en *Bryoria* y *Pseudephebe* (*Parmeliaceae*).

Introducción

Bryoria y *Pseudephebe* son dos géneros de hongos liquenizados que se incluyen dentro del Clado Alectorioide de la familia *Parmeliaceae* (Divakar *et al.* 2015). Aunque su distribución es cosmopolita, siempre crecen ligados a áreas frescas y húmedas, con frecuencia ambientes similares a los boreo-alpinos. *Pseudephebe* es el género con menos especies del clado, con tan solo dos, en principio, morfológicamente diferenciadas, aunque con frecuencia se encuentran ejemplares con características intermedias que imposibilitan una identificación fiable.

Por otra parte, el género *Bryoria,* es con diferencia el más numeroso de entre todos los alectorioides (*c.* 80 especies; Myllys *et al*. 2011a). Su aspecto, bastante homogéneo, con lacinias cilíndricas de apariencia capilar, la relativa simplicidad de sus estructuras vegetativas y, salvo excepciones, la ausencia de caracteres sexuales desarrollados, propicia que las especies de este género sean de difícil identificación (Brodo & Hawksworth 1977). La poca variabilidad morfológica, favoreció que en el pasado la taxonomía basada en la composición de las sustancias que se acumulan formando cristales extracelulares (extrolitos) en sus talos cobrara mucha importancia. Tal es así, que incluso algunas especies se describieron simplemente porque poseían extrolitos distintos a otras. Los análisis moleculares realizados por Myllys *et al*. (2011b) condujeron a la descripción de cinco secciones infragenéricas, de las cuales, *Bryoria* sect. *Implexae* resulto ser la más necesitada de una revisión taxonómica basada en caracteres moleculares. Velmala *et al.* (2014) se centraron en resolver el concepto de especie en esta sección, utilizando para ello tres marcadores moleculares estándar. El resultado reveló la presencia de cuatro linajes, dos de ellos formados por varias especies filogenéticamente entremezcladas. Sin embargo, debido a que las especies tradicionales mostraban combinaciones consistentes de caracteres morfológicos, químicos y en ocasiones ecológicos, y que éstas podían encontrarse creciendo en el mismo micronicho, quedando

descartada así la posibilidad de que sean adaptaciones ambientales, decidieron no sinonimizarlas a la espera de estudios moleculares más exhaustivos.

Objetivos

Los objetivos específicos establecidos en esta tesis doctoral son:

- Testar la utilidad de la autofluorescencia a nivel microscópico para detectar la ubicación de extrolitos u otras sustancias acumuladas (Capítulo 1).
- Averiguar si los extrolitos producidos por los líquenes de *Bryoria* sect. *Implexae* se distribuyen por todo el talo o se acumulan en regiones concretas (Capítulos 1 y 2).
- Explorar la relación entre la composición de los extrolitos y el parentesco genético en individuos geográficamente cercanos y morfológicamente similares (Capítulo 2).
- Obtener marcadores genéticos de alta variabilidad (microsatélites y secuencias intergénicas altamente variables) que permitan realizar un estudio poblacional y filogenético en *Bryoria* sect. *Implexae* (Capítulos 3, 4 y 5).
- Resolver el concepto de especie en *Bryoria* sect. *Implexae*, mediante una aproximación integrativa y utilizando marcadores de ADN altamente variables (Capítulo 4).
- Realizar un estudio poblacional y filogeográfico de *Bryoria fuscescens* agg. en Europa y el Norte de África (Capítulo 5).
- Averiguar las posibles causas que pueden provocar la alta variabilidad fenotípica en *Bryoria fuscescens* agg. (Capítulo 5).
- Comprobar si el hongo liquenícola *Raesaenenia huuskonenii* tiene preferencia por ciertos fenotipos/taxones de *Bryoria* sect. *Implexae*. (Capítulo 5).
- Determinar filogenéticamente la especie a la que pertenecen ejemplares de *Bryoria* sp. recolectados en Chile (Capítulo 6).
- Analizar la adscripción genérica de *Bryoria mariensis*, un taxón con características intermedias entre varios géneros (Capítulo 7).
- Establecer el concepto de especie en el género *Pseudephebe* (Capítulo 7).

Materiales y métodos

Tres son las aproximaciones metodológicas principales que se han usado para tratar de alcanzar los objetivos planteados:

Estudio de los extrolitos. Para desvelar la composición, así como la ubicación de los extrolitos dentro del talo de los líquenes, se realizaron test químicos y cromatografías en capa fina (TLC), utilizando distintas regiones del talo y cuatro sistemas de disolventes. Para una mayor precisión en la ubicación de las sustancias a nivel anatómico se utilizó el microscopio de fluorescencia.

Estudios filogenéticos. Con la finalidad de desvelar el parentesco evolutivo de los especímenes estudiados, cerca de 250 muestras recolectadas principalmente en Europa y América, se obtuvieron datos basados en caracteres químicos, morfológicos, corológicos, marcadores moleculares basados en secuencias estándar (IGS, ITS, GAPDH, MCM7, mtSSU y RPB1), nuevos marcadores moleculares (FRBi13, FRBi15, FRBi16, FRBi18 y FRBi19) y 18 marcadores de microsatélites diseñados específicamente para *Bryoria* sect. *Implexae*. Con estos datos, se realizaron distintos análisis, principalmente: reconstrucción de fenogramas, test de incongruencia entre topologías de árboles, detección de recombinación, reconstrucción filogenética por parsimonia, *neighbour joining*, máxima verosimilitud, inferencia bayesiana, detección de *pools* genéticos por inferencia bayesiana (STRUCTURE), análisis de coordenadas principales, análisis de componentes principales, reconstrucción de redes de haplotipos, test de delimitación de especies (ABGD, PTP, GMYC, DISSECT, …), estudios de inferencia demográfica y datación de la edad de los linajes.

Estudios filogeográficos. El análisis filogeográfico se realizó utilizando 1.400 muestras recolectadas en Europa y el Norte de África. Los especímenes fueron caracterizados tanto morfológica como químicamente. El perfil genético de cada ejemplar se caracterizó utilizando 18 marcadores de microsatélites. Con estos resultados se realizaron numerosos test de diversidad genética (polimorfismo de los distintos loci, diversidad genética no sesgada (uh), medidas de ligamiento entre loci no sesgadas (rBarD), riqueza alélica (AR), riqueza alélica privada (PAR), test de expansión o reducción poblacional, AMOVA, detección de señales de reproducción sexual, análisis de dinámica poblacional, …), tests de búsqueda de estructura genética (STRUCTURE y DAPC principalmente), análisis de aislamiento genético por distancia geográfica, análisis de migración y estimación de la distribución potencial actual y pasada.

Resultados

El género *Pseudephebe* comprende dos grandes linajes bien definidos, sin embargo, estos resultan ser fenotípicamente crípticos. Se designa un epitipo para cada linaje, acomodando las dos especies morfológicas del género al concepto de especie filogenética. La especie *Bryoria mariensis* ha resultado estar anidada dentro de *Pseudephebe minuscula* y por tanto ha sido sinonimizada. Los estudios muestran que las especies de *Pseudephebe* desarrollan pseudocifelas y producen ácido norestíctico, previamente no detectados en este grupo.

Respecto a la taxonomía de *Bryoria* sect. *Implexae*, las reconstrucciones filogenéticas muestran que el grupo incluye cuatro linajes con apenas variabilidad dentro de cada uno de ellos. Los resultados de la taxonomía integrativa nos han llevado a reducir *Bryoria* sect. *Implexae* de once especies a cuatro: *Bryoria fuscescens*, *B. kockiana*, *B. pseudofuscescens* y *B. glabra*. Las tres primeras especies las consideramos crípticas, mientras que *Bryoria glabra*, muestra caracteres sutiles que permiten su identificación. La separación entre las tres primeras especies ocurrió hace alrededor de 100.000 años, por lo que consideramos que se trata del evento de especiación más reciente que se conoce en líquenes. Se designan nuevos epitipos para cada taxon, actualizando las descripciones de cada especie. Dentro de *Bryoria fuscescens*, parece existir un proceso de especiación influenciado por la deriva génica hacia dos fenotipos con altos niveles de reparto incompleto de linajes.

En cuanto a los estudios filogeográficos en Europa *Bryoria fuscescens* incluye tres grandes grupos genéticos, dos de amplia distribución y uno restringido al norte de Escandinavia, el cual muestra caracteres intermedios entre *B. fuscescens* y *B. pseudofuscescens*. La zona de mayor diversidad genética se sitúa en la Península Escandinava, seguida de los Alpes y la Península Ibérica. Los Cárpatos carecen de haplotipos exclusivos. No se detectan evidencias de que los apotecios favorezcan una mayor diversidad genética. A medida que aumenta la distancia geográfica entre las poblaciones estudiadas, no observamos un mayor aislamiento genético entre ellas. Además, se detectan altos niveles de flujos migratorios, especialmente de Escandinavia hacia el Sur de Europa y Norte de África. Estos dos últimos resultados entendemos que están influenciados por la presencia de haplotipos ancestrales compartidos entre las distintas regiones, por lo que sus valores posiblemente estén sobreestimados. Se descarta que la composición de los extrolitos tenga valor taxonómico en este grupo, aunque la presencia de ácido barbatólico y la ausencia de ácido fumarprotocetrárico muestran tendencias evolutivas. Algunos extrolitos parecen estar ligados en parte a factores ambientales.

Conclusiones

1. La fluorescencia se confirma como una herramienta útil para ubicar y en ocasiones identificar metabolitos secundarios acumulados en los talos liquénicos.
2. En *Bryoria fuscescens* agg. tanto la presencia como la composición de los extrolitos puede ser variable en distintas regiones del talo y en ocasiones aparecer asociada a pseudocifelas o soralios.
3. La presencia y composición de los extrolitos en *B. fuscescens* s. l. no está relacionada con el parentesco genético ni con las morfoespecies.
4. Las poblaciones de *Bryoria fuscescens* s. l. en la Región Mediterránea muestran combinaciones de caracteres que no encajan con el concepto de especie establecido a partir de poblaciones boreales.
5. Se han desarrollado marcadores de microsatélites específicos de *Bryoria* sect. *Implexae* que reúnen las características necesarias para ser utilizados en estudios poblacionales.
6. La taxonomía integrativa permite establecer un concepto de especie en *Bryoria* sect. *Implexae* que no revelan taxonomías basadas en solo un tipo de datos. De las 14 morfoespecies analizadas en esta tesis, solo cuatro se mantienen como especies, siendo *B. fuscescens*, *B. kockiana* y *B. pseudofuscescens* crípticas y *B. glabra* sutilmente distinguible.
7. Las especies de *Bryoria fuscescens* agg. constituyen el evento de especiación más reciente conocido en líquenes.
8. La especie *Bryoria fuscescens* s. str. presenta tres grandes grupos genéticos en Europa y Norte de África, dos de ellos de amplia distribución y uno restringido al norte de Escandinavia. Los rasgos genéticos de este último son intermedios entre *Bryoria fuscescens* y *B. pseudofuscescens*.
9. La elevada capacidad de dispersión detectada en *Bryoria fuscescens* s. str. parece ser el resultado de un artefacto producido por haplotipos ancestrales de amplia distribución.
10. La Península Escandinava, seguida por los Alpes y la Península Ibérica, constituyen las zonas con mayor riqueza genética de *Bryoria fuscescens* s. str. en Europa. La diversidad genética de las poblaciones es independiente del desarrollo de apotecios en sus talos.

11. *Bryoria fuscescens* s. str. parece estar sumida en un proceso evolutivo de deriva hacia dos grupos fenotípicos con altos niveles de reparto incompleto de linajes.
12. El hongo liquenícola *Raesaenenia huuskonenii* puede crecer sobre *Bryoria* fuscescens agg. independientemente de la morfoespecie, quimiotipo o grupo genético.
13. Los especímenes de *Bryoria* recolectados en Chile se confirman como una especie nueva propuesta como *Bryoria araucana*.
14. *Bryoria mariensis* debe de considerarse sinónima de *Pseudephebe minuscula*.
15. *Pseudephebe minuscula* es una especie muy variable cuya morfología se solapa con la de *P. pubescens*, por lo que ambas especies deben de considerarse crípticas.

Abstract

Tittle

Species, phylogeography, and extrolite production in *Bryoria* and *Pseudephebe* (*Parmeliaceae*).

Introduction

Bryoria and *Pseudephebe* are two lichen-forming fungal genera included in the Alectorioid Clade of the family *Parmeliaceae* (Divakar *et al.* 2015). Despite its cosmopolitan distribution, its occurrence is mainly linked to clean and humid environments, usually in boreo-alpine habitats. *Pseudephebe* is the smallest genera of the clade, with only two morphologically delimited species. However, specimens with intermediate characteristics are common, impeding a reliable identification.

Contrarily, the genus *Bryoria* is the most numerous among the alectorioids, with *c.* 80 species (Myllys *et al.* 2011a). Its appearance is quite homogeneous, with cylindrical capillary-like lacinae, relatively simple vegetative structures, and, with some exceptions, without sexual reproductive features; making this genus taxonomically complicated (Brodo & Hawksworth 1977). The scarce distinctive features to identify the samples across the genus favoured, in the past, a taxonomy based on the composition of the chemical substances accumulated forming extracellular crystals (extrolites). In fact, some species were described solely based on its extrolite composition. Molecular analyses performed by Myllys *et al*. (2011b) conducted to the description of five infrageneric sections, of these *Bryoria* sect. *Implexae* resulted unresolved and needed a revision. Velmala *et al.* (2014) performed a study focusing on this section and using a higher number of specimens and three standard DNA markers. Results revealed the presence of four lineages; two of them including different species phylogenetically intermixed. However, traditional species showed a consistently observed close agreement between chemical constituents, thallus morphology, and, in some cases, ecological preferences. Moreover, different species can be found growing in the same habitat, discarding environmental adaptations. For this, authors decided to do not synonyms them without more exhaustive molecular analyses.

Objectives

- Test the utility of the autofluorescence at microscopic level to detect the location of the extrolites and other accumulated substances (Chapter 1).
- Find out if extrolites synthesized by *Bryoria* sect. *Implexae* are distributed along all the thallus or are accumulated in certain regions (Chapters 1 & 2).
- Elucidate the relation among extrolite composition and genetic kinship in specimens geographically close and morphologically similar (Chapter 2).
- Design and obtain highly variable genetic markers (microsatellites and DNA sequences) to perform a population and phylogenetic study on *Bryoria* sect. *Implexae* (Chapters 3, 4 & 5).
- Evaluate species boundary in *Bryoria* sect. *Implexae* thorough an integrative approximation and using highly variable DNA markers (Chapter 4).
- Elucidate phylogeographical structure, speciation process and population structure and assess factors triggering high phenotypic variability in *Bryoria fuscescens* agg. in Europe and North Africa (Chapter 5).
- Test if the lichenicolous fungus *Raesaenenia huuskonenii* has preference by certain phenotypes/taxa of *Bryoria* sect. *Implexae*. (Chapter 5).
- Identify using molecular methods the species of enigmatic *Bryoria* specimens collected on Chile (Chapter 6).
- Analyse the generic ascription of *Bryoria mariensis*, a taxon with intermediate characteristics within different genera (Chapter 7).
- Investigate species boundary and species concept in the genus *Pseudephebe* (Chapter 7).

Materials and methods

The planned objectives were resolved thorough three different methodological approximations:

Extrolite studies: To reveal the extrolite composition, as well as its location inside the lichen thallus, spot tests, and thin layer chromatographies were done using different thallus

regions and four solvent systems. Additionally, the fluorescence microscopy was used for a better resolution to locate the extrolites at anatomic level.

Phylogenetic studies: To reveal the genetic kinship of the studied specimens, around 250 samples collected mainly in Europe and North America were analyzed using chemical, morphological, and corological characters, standard DNA sequences (IGS, ITS, GAPDH, MCM7, mtSSU y RPB1), newly generated markers (FRBi13, FRBi15, FRBi16, FRBi18 and FRBi19), and 18 microsatellites markers specifically designed for *Bryoria* sect. *Implexae*. With these data different analyses were performed, mainly: phenogram reconstruction, topological incongruence tests, recombination detection, parsimony, neighbour joining, maximum likelihood and Bayesian phylogenetic reconstruction, Bayesian inference for genepools detection (STRUCTURE), principal coordinate analyses (PCoA), principal components analyses (PCA), haplotype network reconstruction, species delimitation tests (ABGD, PTP, GMYC, DISSECT), past demographic estimation tests and node ages estimation tests.

Phylogeographical studies: The phylogeographycal analysis was performed with 1400 samples collected around Europe and North Africa. Specimens were morphologically and chemically characterized. The genetic relations among specimens were tested using 18 microsatellite markers. With this data different tests of genetic diversity were performed as (polymorphism of each locus, unbiased genetic diversity (uh), unbiased levels of linkage disequilibrium (rBarD), allelic richness (AR), private allelic richness (PAR), population expansion and reduction tests, AMOVA, tests to detect sexual reproduction), genepools detection tests (STRUCTURE and DAPC), isolation by distance tests, migration analyses, and potential of present and past distribution.

Results

The genus *Pseudephebe* is composed by two well isolated phenotypically cryptic lineages. In order to facilitate taxonomic identification an epitype was designated for each one, establishing a phylogenetic species concept for each traditional species. *Bryoria mariensis* was synonymized with *Pseudephebe minuscula*. Additionally, our studies reveal that *Pseudephebe* can have pseudocyphellae and produce norstictic acid; these were never reported before in the literature.

The phylogenetic reconstructions in *Bryoria* sect. *Implexae* showed that the group contains four main clades closely related. The results of the integrative taxonomy concluded to reduce from eleven species to four: *Bryoria fuscescens*, *B. kockiana*, *B. pseudofuscescens*

and *B. glabra*. The three first species are considered as cryptic, whereas *Bryoria glabra* shows subtle morphological differences that sometimes allow its identification. The divergence among the three first species is estimated around 100 000 years ago, being the most recent speciating event among all known lichens so far. New epitypes were designated for each taxon, and species descriptions were adapted to the new concept. In *Bryoria fuscescens*, there are signals of a speciating process influenced by genetic drift and high levels of incomplete lineage sorting.

The phylogeographical studies on *Bryoria fuscescens* showed the presence of three main genepools, two widely distributed and one restricted to North Scandinavia, showing intermediate genetic information between *B. fuscescens* and *B. pseudofuscescens*. The highest genetic diversity was detected in Scandinavia, followed by Alps and Iberian Peninsula. The Carpathians lacked private alleles. The classic thought that apothecia increases the genetic diversity of a population has not been found. Isolation by distance tests revealed that geographically distant populations are not significantly genetically isolated. High levels of migration were detected, especially from Scandinavia to southern regions. These two results seems influenced by the presence of shared ancestral polymorphisms which could be increasing the estimation. Extrolite composition is discarded as a taxonomically informative character, whereas the presence of barbatolic acid and the absence of fumarprotocetraric show evolutionary trends. Some extrolites seems linked to environmental factors.

Conclusions

1. The fluorescence is confirmed as a useful tool to locate and sometimes identify the secondary metabolites accumulated in the lichen thalli.
2. In *Bryoria fuscescens* agg, the presence and composition of extrolites can be variable among different thallus regions and sometimes can appear linked to pseudocyphellae or soralia.
3. The presence and composition of extrolites in *B. fuscescens* s. l. is neither related with genetic affinity nor morphospecies.
4. The populations of *Bryoria fuscescens* s. l. in the Mediterranean region show a combination of characters that does not fit with the established morphospecies concept based on boreal specimens.
5. New microsatellite markers specific for *Bryoria* sect. *Implexae* have been designed, containing the necessary requisites to perform phylogeographical studies at population level.

6. Integrative taxonomy allows to develop a species concept in *Bryoria* sect. *Implexae* that cannot be obtained using a single approach. Of the 14 morphospecies analyzed, only four accomplish with the phylogenetic species concept, being *B. fuscescens*, *B. kockiana* and *B. pseudofuscescens* cryptic and *B. glabra* subtly distinguishable.
7. The species of *Bryoria fuscescens* agg. are revealed as the most recent speciation event known so far in lichens.
8. *Bryoria fuscescens* s. str. contains three main genepools in Europe and North Africa, two of them widely distributed, whereas one is restricted to north of Scandinavia. The genetic traits of the last one are intermediate between *Bryoria fuscescens* and *B. pseudofuscescens*.
9. The high dispersal capacities of *Bryoria fuscescens* s. str. detected here seems influenced by the presence of shared ancestral polymorphisms.
10. In the European region, the Scandinavian Peninsula, followed by Alps and Iberian Peninsula are the areas with richest genetic diversity in *Bryoria fuscescens* s. str. The genetic diversity of the populations does not correlate with the presence or absence of apothecia.
11. *Bryoria fuscescens* s. str. seems involved in an evolutionary process influenced by genetic drift towards two phenotypic groups with high levels of incomplete lineage sorting.
12. The lichenicolous fungus *Raesaenenia huuskonenii* grows on *Bryoria fuscescens* agg. independently of the morphospecies, chemotype or genepool.
13. The *Bryoria* specimens collected in Chile belongs to an undescribed species here proposed as *Bryoria araucana*.
14. *Bryoria mariensis* should be considered a synonym of *Pseudephebe minuscula*.
15. *Pseudephebe minuscula* is a very variable species with a phenotype overlapped with *P. pubescens*, and for this, both species should be considered cryptic.

Introducción

Usnea trichodeoides **creciendo sobre el árbol** ***Hypericum revolutum*****, Monte Meru, Tanzania. (foto: C. G. Boluda).**

Introducción

La complejidad del concepto de especie

El concepto intuitivo de especie encaja con una visión literal de las religiones abrahámicas (judía, cristiana y musulmana), que surgieron a partir de los mensajes difundidos por tres profetas hipotéticamente enviados por un mismo Dios. Este único ente creador instauraría las propiedades de cada organismo vivo, formando grupos de individuos que compartirían características entre sí, pero no con otros. Dado que la morfología de la descendencia no se debía desviar significativamente de la de los padres, las formas creadas permanecerían inalteradas con el tiempo. El término especie, empezó a establecerse en una época y región en la que esta opinión era la mayoritaria, influyendo significativamente en su concepto científico actual. Hoy en día, la ciencia, a través de multitud de sólidos datos, descarta que los organismos se hayan generado espontáneamente y se hayan mantenido inalterados, siempre con las mismas características a lo largo del tiempo. Todo apunta a que la gran variabilidad de formas de vida existente son el producto de la acumulación gradual de cambios, a partir de un punto inicial de reacciones químicas autoreplicativas. En un gradiente continuo de morfologías o linajes genéticos, establecer barreras es impracticable. Pero la muerte de los organismos conlleva a la extinción de linajes, transformando un gradiente fenotípico o genotípico continuo en discontinuo, y por tanto divisible, dando sentido a un concepto evolutivo de especie. Desde el punto de vista evolutivo estricto, se podría decir que las especies no existen. Imaginemos que tuviésemos una reconstrucción de linajes que incluyera a todos los individuos que hayan existido, dividir estos grupos en especies sería impracticable. No obstante, nunca se ha llegado a adquirir tal nivel de conocimiento, lo que propicia que un concepto de especie tenga sentido, o al menos utilidad práctica.

Pese a que la percepción de lo que podría ser una especie parece obvia, continúa habiendo intensos debates sobre cómo debería definirse. La amplia diversidad de formas de vida, tanto actuales como fósiles, ha propiciado que se reconozcan hasta 26 definiciones conceptuales de especie (Wilkins 2011). Uno de los conceptos más utilizados y que mejor encaja con los datos obtenidos a través de la biología molecular es el que formuló de Queiroz (2007), que define a una especie como un linaje formado por un conjunto de poblaciones (una metapoblación) que evoluciona independientemente a otras. Pero el principal problema a la hora de interpretar una especie, según este concepto, suele aparecer al intentar establecer los límites de esta con otras emparentadas.

La noción de especie nació en un momento en el cual no se conocían, o no se aceptaban los fundamentos de la evolución de los organismos, por lo que se aplicaba siguiendo principalmente el sentido común. Actualmente se sabe que los organismos están en continuo cambio, si bien no siempre a nivel morfológico, si a nivel molecular. Con el acúmulo de estos cambios, una especie tendería a formar todos los fenotipos posibles, pero la selección natural elimina aquellos que sucumben antes de originar descendencia. Esto va perfilando las características de la descendencia, formando linajes que pueden ser rastreados. A pequeña escala, estos linajes aparecen como redes, al menos en los organismos con reproducción sexual, pero a gran escala, como líneas que se ramifican. Las características fenotípicas de cada una de estas líneas pueden quedar estancadas ante ciertas condiciones de selección natural, formando en este caso especies conceptualmente sólidas. Pero del mismo modo, pueden estar cambiando, formando linajes constantemente, impidiendo que el concepto de especie pueda ser aplicado objetivamente. No hay que olvidar que la evolución es como una película en la que nosotros solo vemos el último fotograma, tanto en los organismos morfológicamente “estables” como en los que están especiando. En la práctica, una visita a un espacio natural nos muestra que las distintas morfologías de los seres vivos están bien delimitadas, formando grupos que comparten características, en gran parte, debido a una intensa presión de la selección natural. Sin embargo, la inmensa mayoría de formas de vida no son visibles a nuestros ojos, son microscópicas, generalmente asexuales, tienen ciclos de vida rápidos y la selección natural no suele actuar con la presión necesaria como para dar lugar a especies morfológicamente tan bien delimitadas como las de los organismos macroscópicos. Podemos decir, atendiendo a lo anterior, que la mayor proporción de la diversidad biológica quedaría englobada dentro de las bacterias y arqueas, y, en mucha menor medida, en eucariotas microscópicos (Hug *et al.* 2016). En estos organismos, y a medida que se descubren miles de nuevos taxones, son frecuentes las reconstrucciones filogenéticas que muestran una infinidad de linajes imbricados que muchas veces nos impidienden establecer límites objetivos entre ellos, ya sea de rango de especie o superior. A todo esto, se suman eventos de transferencia horizontal de ADN, que transforma los linajes lineales en redes (Kunin *et al.* 2005). El concepto de especie puede ser variable en las distintas ramas del árbol de la vida y ha de ser ajustado a cada grupo. Mientras que en un vertebrado se sigue un concepto muy restrictivo, en una bacteria, como en el caso de la conocida especie *Escherichia coli*, podemos encontrar tanta variabilidad genética como la que existe entre un hombre y una lombriz (Lukjancenko *et al.* 2010).

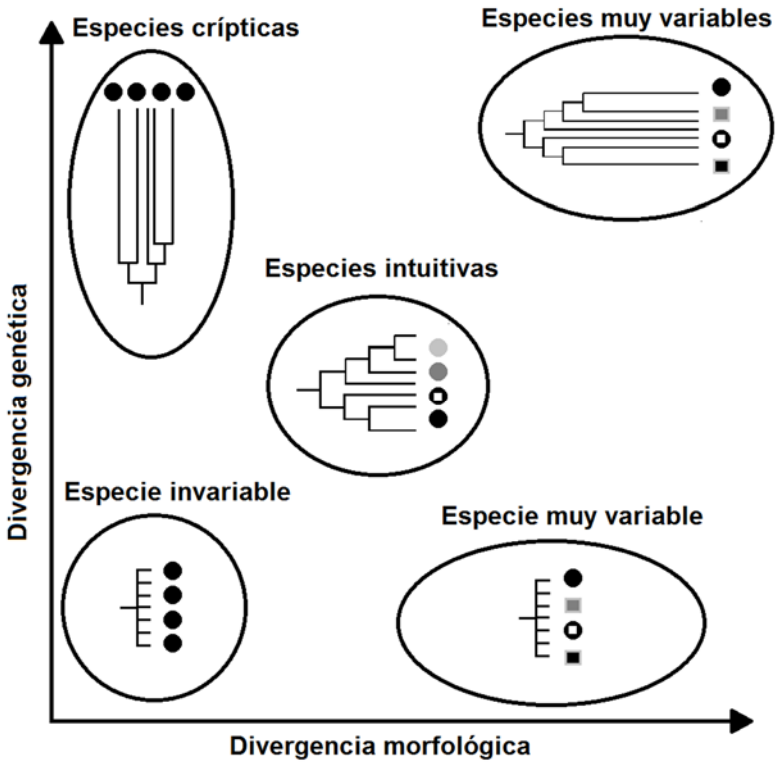


Fig. 1. Representación esquemática de probables alternativas que puede ofrecer la interacción entre la variabilidad genética y la morfológica de los taxones (especies) a la hora de delimitar especies (representación: C. G. Boluda).

En el caso de los hongos, actualmente, se conocen más especies macroscópicas que microscópicas, pero esto parece que no seguirá así por mucho tiempo. El metagenoma estudiado de determinados suelos o sedimentos muestra gran cantidad de linajes de hongos todavía desconocidos, aún por describir (Jones *et al.* 2011). Puesto que los hongos microscópicos son fenotípicamente simples, y además variables dependiendo del medio en el que crecen, se aproxima un futuro en el que podrán ser definidos, con toda probabilidad, una gran cantidad de taxones morfológicamente indistinguibles. Esto muestra que los hongos microscópicos, incluidos los hongos filamentosos, en ocasiones se comportan filogenéticamente como los procariotas, ya que su morfología no parece jugar un papel importante para el proceso de selección natural. Ya existen casos en los que una especie de hongo ha sido descrita basándose solo en secuencias de ADN, sin que los especímenes hayan sido "vistos" o detectados, más allá que con datos moleculares (de Beer *et al.* 2016). Esta práctica es bastante habitual en procariotas, donde se han descrito gran cantidad de linajes en los que nunca se ha tenido la oportunidad de observar ningún ejemplar (Kozubal *et al.* 2012). Cada vez más, se detectan grupos de hongos claramente aislados genéticamente, pero que carecen de morfología asociada. En los últimos años, se ha podido observar que

esto no solo ocurre en hongos con morfologías simples, sino también en hongos macroscópicos como los que producen setas o en los líquenes (hongos liquenizados). El concepto de especie críptica o criptoespecie (Sáez & Lozano 2005) se aplica en los casos en que las especies son fenotípicamente indistinguibles, pero si lo son molecularmente (Fig. 1). En ocasiones un estudio detallado puede revelar sutiles caracteres que permiten distinguir ambos taxones, pasando a llamarse entonces especies pseudocrípticas (Medina *et al.* 2012). Si para distinguir estos taxones hacen falta caracteres ajenos al individuo, como ecológicos o de distribución, entonces hablamos de especies semicrípticas (Vondrák *et al.* 2009). Finalmente, el concepto de especies hermanas (*sibling species*), íntimamente relacionado con el de especies crípticas, tiende a aplicarse para el caso de especies crípticas que son filogenéticamente hermanas, es decir, comparten un ancestro común inmediato (Bickford *et al.* 2007). También se da el caso contrario al de las especies crípticas, en el que morfologías claramente delimitadas han resultado estar incluidas en un mismo linaje, es decir, son conespecíficas (Leavitt *et al.* 2011). En tal caso, se suele decir que éstas son morfoespecies (Fig. 2). En ocasiones, las morfoespecies se diferencian principalmente en sus estrategias reproductivas, siendo una asexual mientras que la otra se reproduce sexualmente. En este último caso, generalmente se habla de pares de especies (Du Rietz 1924; Crespo & Pérez-Ortega 2009), aunque esté concepto incluye más variables.

Fig. 2. Las morfoespecies *Usnea subfloridana* (izquierda, esteril) y *U. florida* (derecha, fértil) no están filogenéticamente aisladas, pero ambas muestran combinaciones de caracteres consistentes con el concepto morfológico de especie (fotos: C. G. Boluda).

Como se menciona en el siguiente apartado, un liquen es una comunidad de organismos que viven en una simbiosis, formada por al menos un hongo y un alga. Dado que la parte fúngica cobra una mayor importancia estructural y funcional, el nombre específico de esta comunidad viene dado por la especie de hongo mayoritaria. Los líquenes son organismos relativamente simples, en los que encontrar caracteres morfológicos diagnóstico que no se solapen con los de otras especies puede, en ocasiones, ser muy complicado. Muchos de los conceptos de especie existentes no se pueden aplicar a los hongos y, en particular, a los liquenizados. Por ejemplo, el aislamiento reproductivo se considera una de las principales propiedades de una especie si seguimos un concepto biológico (Dobzhansky 1935; Mayr 1942), pero muchos de estos hongos son asexuales, o por su naturaleza, no se puede experimentar con ellos, hasta el momento, procesos de hibridación. El concepto de especie morfológica (Cronquist 1978) en ocasiones no se puede aplicar, ya que, por ejemplo, la morfología desarrollada por la parte fúngica cambia radicalmente en presencia o ausencia del fotobionte e incluso dependiendo de qué fotobionte tenga, originándose en este caso lo que se denominan fotosimbiodemos o fotomorfos (Magain *et al.* 2012). Posteriormente y para intentar solventar estos problemas de falta de distinción, a los caracteres morfológicos se sumaron los químicos, pero, aun así, las combinaciones resultantes son insuficientes para caracterizar todos los linajes evolutivos que se reconocen (Crespo & Pérez-Ortega 2009). Por esto y cada vez con más frecuencia, se utilizan los caracteres moleculares como principal o único rasgo que defina a un taxon en el rango de especie, ya que suelen ser más objetivos y fiables que los fenotípicos. Estos caracteres moleculares permiten detectar linajes evolutivos y barreras reproductivas en grupos morfológicamente similares mediante la comparación de regiones de ADN homólogas. Hay muchas metodologías que permiten comparar y analizar las regiones de ADN. Originalmente fueron muy utilizadas las metodologías de máxima parsimonia y *neighbour joining*, con la mejora de los ordenadores, se pasó a la inferencia bayesiana o a la máxima verosimilitud, y parece que actualmente está cobrando fuerza el análisis basado en la coalescencia (Rannala & Yang 2003).

Sin embargo, obtener árboles filogenéticos con linajes claramente definidos que puedan corresponderse con especies, no significa que éstos expresen la verdadera historia de las mismas. Esto es especialmente evidente en eventos de radiación reciente o en linajes en especiación. Procesos naturales como la hibridación o la recombinación de genes conducen a incongruencias entre las distintas topologías de los distintos marcadores. A esto ha de sumarse el reparto incompleto de linajes (*incomplete lineage sorting*), en el cual cada marcador, al haber acumulado distintas mutaciones, puede inferir un árbol filogenético topológicamente incompatible con los de otros marcadores (Fig. 3). En estos casos, taxones fenotípicamente bien caracterizados pueden aparecer como parafiléticos o polifiléticos en una

reconstrucción filogenética (Saag *et al.* 2014). Efectivamente, siguiendo el concepto filogenético de especie, esto llevaría a considerar su conespecificidad, por tanto su probable sinonimización, aunque "realmente" fueran especies diferentes. En estos casos, el árbol de genes no se correspondería con la historia real de las especies, siendo necesarios otro tipo de datos y análisis adicionales para comprobar la validez de las morfoespecies.

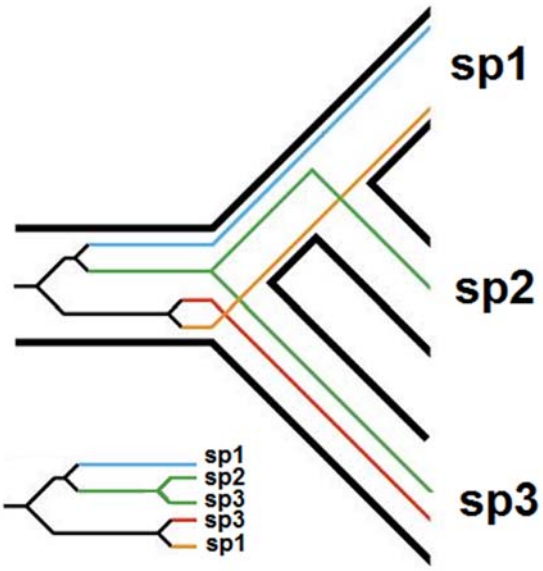


Fig. 3. Ejemplo de reparto incompleto de linajes. La topología dibujada en negro representa las relaciones evolutivas reales entre las tres especies. En su interior, en color, aparece la topología de un marcador molecular con el fenómeno de reprato incompleto de linajes. En el árbol inferior se representan las relaciones evolutivas que este marcador inferiría (representación: C. G. Boluda).

El concepto de especie utilizado en esta tesis es el mayoritariamente utilizado en las más actuales aportaciones taxonómicas sobre líquenes (de Quieroz 2007; Altermann *et al.* 2014; Leavitt *et al.* 2013), donde una especie se considera un linaje metapoblacional que evoluciona de forma independiente. Sin embargo, como se explica arriba, corroborar que un linaje evoluciona de forma independiente puede ser un trabajo difícil y lleno de artefactos confusos. Para maximizar la objetividad a la hora de situar la barrera interespecífica en una reconstrucción filogenética, en la presente tesis se aborda el concepto de especie desde un punto de vista integrativo (Dayrat 2005).

El liquen como un ecosistema

Aunque tradicionalmente a los líquenes se les ha estudiado como organismos, cada talo liquénico es, en sí mismo, un verdadero ecosistema, en el cual conviven taxones de muy distintos grupos filogenéticos que contribuyen a las propiedades finales del liquen. Un liquen está compuesto por un hongo, el llamado micobionte, y un alga, que recibe el nombre de fotobionte. El fotobionte suele estar incluido en diferentes grupos de algas verdes del filo

Chlorophyta, formando los que se conocen como clorolíquenes. Pero, algunas especies o linajes completos de líquenes, utilizan a cianobacterias (filo *Cyanobacteria*), formando los cianolíquenes. Existen particularidades en las que el talo liquénico incluye como fotobionte a representantes de las algas pardas (filo *Heterokontophyta*; Tschermak-Woess 1988; Gärtner 1992). Se conocen más de 40 géneros de algas simbiontes (Tschermak-Woess 1988; Büdel 1992), pero con diferencia, los más comunes son *Trebouxia*, *Trentepohlia* (algas verdes) y *Nostoc* (cianobacterias). No es raro que una especie de liquen incluya más de una especie de alga, ya sean del mismo grupo, como ocurre por ejemplo en *Ramalina farinacea*, que puede tener dos especies de *Trebouxia* distintas (Casano *et al*. 2011), o de grupos completamente distintos. En este último caso, lo más común es que un cloroliquen tenga también cianobacterias, pero solo en ciertas partes de su talo, formando estructuras diferenciadas, frecuentemente de aspecto tumoroso, que reciben el nombre de cefalodios. En ocasiones, los cefalodios pueden vivir independientemente del resto del liquen, con lo que podríamos decir que un cloroliquen pasa a formar un cianoliquen, dependiendo de si se relaciona con cianobacterias o clorófitos unicelulares. Puesto que las cianobacterias son capaces de fijar nitrógeno atmosférico, esta dualidad le permite al micobionte tener aportes extra de nutrientes. Un liquen se describe como una simbiosis mutualista, definición en gran parte apoyada en que ni el hongo ni el alga suelen encontrarse de forma aislada en el medio. Generalmente la parte fúngica es la mayoritaria, englobando a las algas en su interior, pero en algunas especies, el alga domina sobre el hongo. Por ejemplo, en algunas especies del género *Coenogonium*, (Fig. 4), el hongo cubre exteriormente a los filamentos del alga y es, al menso a simple vista, dificilmente observable si no fuera por la formación de apotecios por parte del hongo. En otros casos, como en algunas especies del género *Collemopsidium*, la simbiosis mutualista a pasado a ser parasitaria, como en *Collemospidium pelvetiae*, que forma peritecios sobre el alga parda marina *Pelvetia canaliculata*. Otro caso curioso de simbiosis entre un hongo y un alga es el de *Geosiphon*, un hongo del filo *Glomeromycota* que posee cianobacterias simbiontes falsamente intracelulares (Gehrig 1996). Debido a que en este caso la simbiosis ocurre a nivel “intracelular”, *Geosiphon* no se considera un hongo liquenizado,

Fig. 4. potecios de *Coenogonium interplexum* entre filamentos de su fotobionte *Trentepohlia* sp. Tailandia (foto: C. G. Boluda).

sino otra forma alternativa de simbiosis. Esto reduce los hongos liquenizados al subreino *Dikarya* (*Ascomycota* + *Basidiomycota*; Hibbett *et al.* 2007).

El fotobionte de los líquenes puede estar incluido dentro de diferentes grupos de organismos, aunque, como hemos dicho, son relativamente pocos los géneros que liquenizan. Sin embargo, la diversidad del micobionte, pese a estar reducida a los *Dikarya*, es muy variada, con unas 20.000 especies conocidas (Lücking *et al.* 2016). Los hongos liquenizados son extraordinariamente polifiléticos, lo que remarca la gran ventaja evolutiva de la liquenización, que parece haber actuado como una potente fuerza evolutiva en la formación de nuevos linajes de hongos (Lutzoni 2001). La capacidad de liquenizar ha surgido de forma independiente en diversos linajes, que, a su vez, pueden haber revertido a un estilo de vida no liquenizado, para posteriormente volver a liquenizar (Lücking *et al.* 2016). Dentro del filo *Ascomycota*, la clase *Lecanoromycetes* destaca por ser la que más especies liquenizadas incluye. Sin embargo, se reconocen 6 clases más, completa o parcialmente liquenizadas: *Arthoniomycetes*, *Coniocybomycetes*, *Dothideomycetes*, *Eurotiomycetes*, *Lichinomycetes* y *Sordariomycetes* (Fig. 5). En el filo *Basidiomycota*, el número de grupos liquenizados es mucho menor y se restringe a la clase *Agaricomycetes* (órdenes *Agaricales*, *Athelliales*, *Cantharellales*, *Corticiales*, y *Lepidostromatales*; Lücking *et al.* 2016).

A parte del micobionte y del fotobionte, los líquenes esconden gran cantidad de bacterias en sus talos. Algunas simplemente se encuentran aquí como especies de amplio espectro, pero otras parecen estar estrechamente ligadas a la simbiosis liquénica (Hodkinson & Lutzoni 2009). El principal grupo de bacterias que participa en esta relación es el de las alfaproteobacterias (filo *Proteobacteria*, clase *Alphaproteobacteria*), aunque muchos otros grupos también pueden ser abundantes (Cardinale *et al.* 2008). La mayor parte de estos microbios no han podido ser cultivados, lo que podría sugerir una estrecha relación entre estas bacterias y la simbiosis liquénica (Cardinale *et al.* 2006). Existen comunidades bacterianas específicas de algunos grupos de líquenes (Grube *et al.* 2009; Bates *et al.* 2011), y otras relacionadas con su distribución o con el tipo de fotobionte del liquen (Hodkinson *et al.* 2012). Las comunidades microbianas, además, pueden variar dependiendo de la edad del talo, el sustrato o el grado de exposición al sol (Cardinale *et al.* 2012). Se desconoce la incidencia de las relaciones microbianas en la estabilidad de la simbiosis liquénica, pero parece ser, al menos en algunos grupos, que podrían actuar como agentes amortiguadores frente a cambios ambientales. Algunas de las funciones que se les atribuye son las de proporcionar un suplemento de nitrógeno orgánico, facilitar la lisis de partículas o sustratos y sintetizar moléculas orgánicas variadas, incluyendo hormonas y sustancias bactericidas (Grube & Berg 2009).

A parte de la simbiosis mutualista entre el micobionte, el fotobionte y las comunidades microbianas asociadas, un liquen puede asociarse con otros organismos con los que vive en simbiosis comensalista o en simbiosis parasitaria. Son muchos los artrópodos que se alimentan y viven en los líquenes, por ejemplo, ácaros o larvas de lepidópteros, que suelen especializarse en ciertos taxones, desde grandes líquenes fruticulosos como *Alectoria*, del que se alimenta la larva de *Alcis jubata*, hasta crustáceos como *Diploicia*, de los que se nutre *Nyctobrya muralis* (Robinson *et al.* 2010). Sin embargo, los simbiontes secundarios más llamativos y más especializados son otros hongos. El término hongo liquenícola se utiliza para designar a todos aquellos hongos que viven encima o dentro de los líquenes, ya sean patógenos, saprótrofos o comensales (Fig. 5). Se conocen más de 1.800 especies de hongos liquenícolas, pero con el inicio de su estudio a nivel molecular, se espera que su número aumente considerablemente (Lawrey & Diederich 2003). Más del 95 % de las especies descritas se incluyen dentro del filo *Ascomycota*, (clases *Arthoniomycetes*, *Dothideomycetes*, *Eurotiomycetes*, *Lecanoromycetes*, *Lichinomycetes*, *Leotiomycetes* y *Sordariomycetes*) quedando restringidas el resto al filo *Basidiomycota* (clases *Agaricostilbomycetes*, *Cystobasidiomycetes*, *Tremellomycetes*, y *Agaricomycetes*). No obstante, para muchas de las especies morfológicamente descritas se desconoce su posición filogenética. Mientras que algunos hongos liquenícolas presentan haustorios que parasitan a las hifas del micobionte (Pippola & Kotiranta 2008), otros parecen nutrirse a partir de las algas simbiontes, algunos empiezan su vida como parásitos o comensales para posteriormente liquenizarse utilizando incluso a la misma especie de alga que el liquen hospedante (Friedl 1987). Finalmente, otros simplemente degradan partes muertas del talo. Puede haber hongos liquenícolas, o incluso parásitos, que no afectan negativamente al liquen y con frecuencia estos ni siquiera muestran síntomas (Millanes *et al.* 2014), mientras que otros pueden llevar a su muerte. Algunos hongos liquenícolas son a su vez parasitados por otros hongos hiperparásitos (Lindgren 2015). La diferencia fisiológica entre hongo liquenizado y hongo liquenícola parece ser en ocasiones tan sutil, que algunos géneros filogenéticamente bien definidos incluyen ambos estilos de vida (Divakar *et al.* 2015; 2017 en revisión).

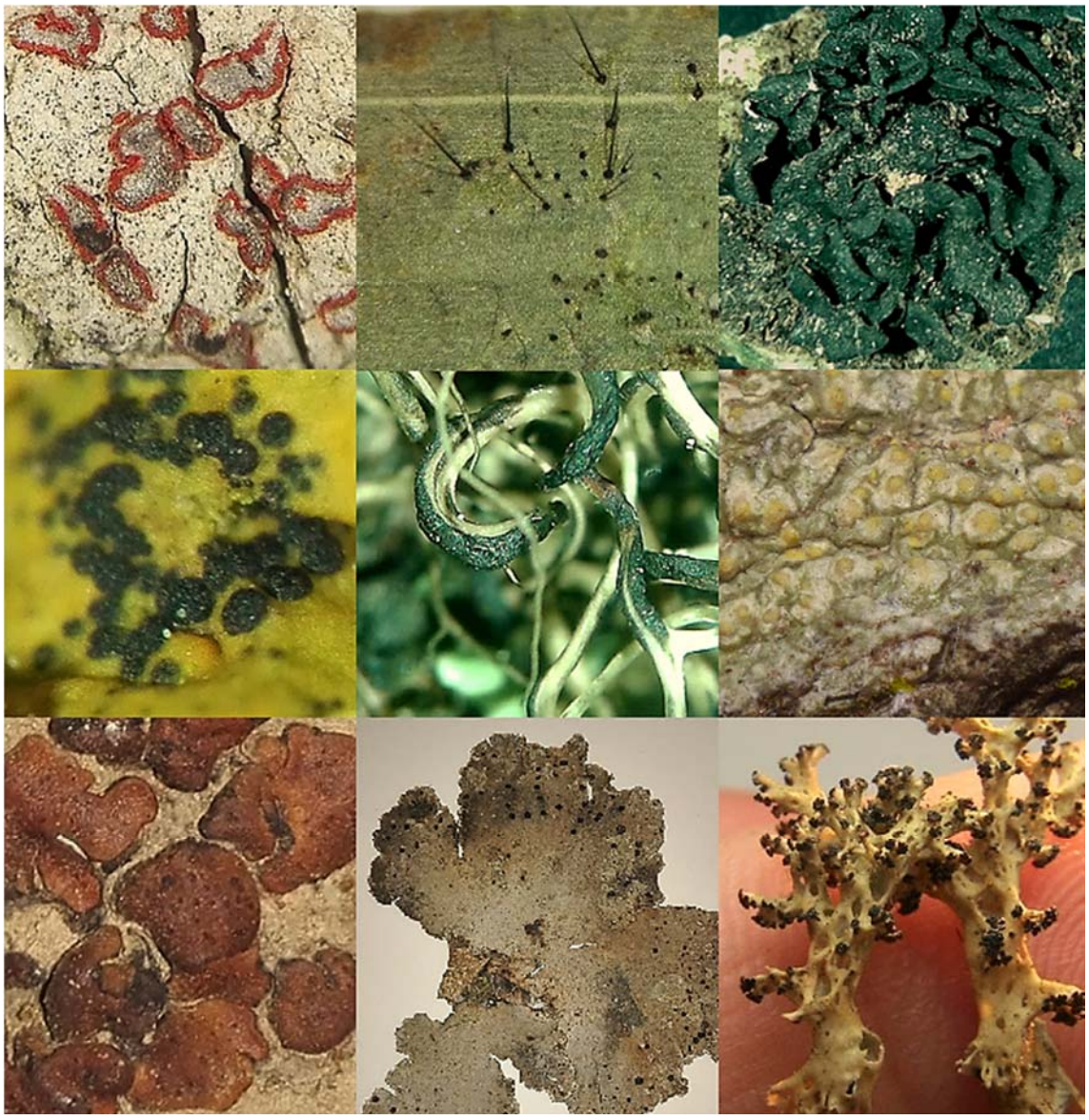


Fig. 5. Ejemplos de la diversidad de líquenes y hongos liquenícolas*. Por columnas, empeando desde la izquierda: *Arthonia cinnabarina* y *A. parietinaria** (*Arthoniomycetes*, España), *Placidium subrufescens* (*Eurotiomycetes*, España), *Tricharia* cf. *vainioi* (liquen foliicola, *Lecanoromycetes*, Tailandia), *Raesaenenia huuskonenii** (*Lecanoromycetes*, Noruega), *Sticta umbilicariiformis* (*Lecanoromycetes*, Tanzania), *Gonohymenia cribellifera* (*Lichinomycetes*, España), *Trypetheliaceae* (*Dothideomycetes*, Tailandia), *Cladia agreggata* s. l. (*Lecanoromycetes*, Australia) (fotos de la colección privada de C. G. Boluda).

Aunque algunas especies son generalistas, los hongos liquenícolas suelen ser específicos de una sola especie de liquen o al menos de un grupo de especies estrechamente emparentadas. En relación a los grupos de líquenes que estudiamos en esta memoria, diversos hongos liquenícolas pueden vivir sobre especies del Clado Alectorioide (*Parmeliaceae*), como *Echinothecium aerophilum* o *Endococcus alectoriae*, en algunas especies de *Alectoria*; *Lichenoconium christiansenii* en *Nodobryoria abbreviata*; o *Abrothallus*

bryoriarum, *Lichenostigma maureri*, *Opegrapha bryoriae*, *Raesaenenia huuskonenii* (parasitado a su vez por *Tremella huuskonenii*) y *Sphaeropezia bryoriae* en *Bryoria* sección *Implexae*.

A parte del micobionte, el fotobionte, las comunidades microbianas y los hongos liquenícolas, recientemente ha cobrado importancia la presencia de levaduras simbióticas del filo *Basidiomycota* como otro probable componente de la simbiosis liquénica (Spribille *et al.* 2016). Estas levaduras se incluyen en la clase *Cystobasidiomycetes* (filo *Basidiomycota*) y viven, por ejemplo, en los líquenes alectorioides. En *Bryoria*, parece que existe una relación entre la presencia de estas levaduras y la cantidad de metabolitos secundarios acumulados en el talo. Puesto que dentro de los *Cyphobasidiomycetes* hay especies liquenícolas, todavía es pronto para decir si estas levaduras son esenciales para mantener la simbiosis liquénica o simplemente son un componente liquenícola más.

Con mucha probabilidad, a todos los organismos mencionados arriba, haya que sumarles una extensa comunidad de protozoos, nuevos linajes bacterianos y gran cantidad de virus, todos ellos ligados de una manera u otra a la simbiosis liquénica.

Sustancias liquénicas

Los líquenes se caracterizan también por su remarcable capacidad de producir extrolitos (también llamados compuestos liquénicos o ácidos liquénicos), que son metabolitos secundarios no solubles en agua que se acumulan formando cristales extracelulares. Al menos el 60 % de las especies de líquenes europeas contienen extrolitos detectables por metodologías simples (Orange *et al.* 2010). Hay tres métodos generalizados para detectar la composición de estas sustancias en los talos liquénicos: aplicando reactivos químicos al liquen, lo que puede o no producir cambios de color en presencia de ciertos extrolitos (test químicos), mediante una cromatografía en capa fina (TLC), o mediante una cromatografía líquida de alta eficacia (HPLC). Los extrolitos a veces se acumulan en regiones concretas del liquen, probablemente dependiendo de su función. Estas sustancias pueden actuar de protectores contra la radiación solar, de antioxidantes, como sustancias hidrófobas que facilitan la aireación o dispersión de propágulos, como disuasores de hongos parásitos, o bien como sustancias tóxicas que evitan su ingestión por animales (Solhaug & Gauslaa 1996; Huneck 1999; Rancan *et al.* 2002; Rubio *et al.* 2002; Werth *et al.* 2013).

La relativa facilidad con la que estos compuestos pueden estudiarse ha propiciado que sean utilizados como un carácter taxonómico muy útil. La importancia que se le adjudicaba a

los extrolitos en el pasado era tal, que gran cantidad de especies de líquenes fueron descritas basándose solamente en su composición diferencial en extrolitos (Brodo & Hawksworth 1977; Park 1985). La taxonomía basada en estos compuestos se ha llamado quimiotaxonomía. En ocasiones, una especie puede desarrollar variaciones en cuanto a la presencia o la composición de alguno de los compuestos liquénicos, aplicandose el nombre de quimiotipo para cada variante. Con frecuencia un metabolito secundario va asociado a otro químicamente relacionado, originado por pasos anteriores o posteriores de la misma ruta metabólica o por degradación parcial espontánea. Cuando varía la proporción entre estos metabolitos, se dice que los diferentes especímenes con estas variaciones pertenecen al mismo quimiosíndrome. Si bien es cierto que la quimiotaxonomía ha sido extensamente utilizada en el pasado, actualmente, con el surgimiento de la filogénia molecular, se ha comprobado que no siempre existe una relación entre composición en extrolitos y parentesco filogenético. No obstante, este continúa siendo un carácter muy importante, y para algunas especies filogenéticamente delimitadas, es el mejor carácter fenotípico que permite distinguirlas.

Los extrolitos son producidos exclusivamente por el micobionte. Generalmente se trata de compuestos formados por un esqueleto de unos pocos anillos aromáticos con diferentes grupos radicales, unidos por enlaces ester, éter o de carbono-carbono. Son varias las rutas metabólicas que producen compuestos liquénicos, entre las que destaca la ruta del acetil-malonil, del ácido shikímico y del ácido mevalónico (Culberson & Elix 1989; Huneck 2001). Los principales extrolitos mencionados en esta tésis se originan a través de la ruta del acetil-malonil, en la cual se toma acetil-coenzima A y malonil-coenzima A y se produce ácido β-orselínico y sus derivados, que terminan transformándose en *para*-dépsidos y finalmente en los metabolitos secundarios específicos. Las enzimas policétido sintasa (PKS) juegan un papel clave en esta ruta. Estas enzimas contienen dominios del tipo cetosintasa, aciltransferasa, cetoreductasa, dehidratasa, enoilreductasa, o portadores de grupos acilo, entre otros. Los genes codificantes de las enzimas PKS se sitúan uno detrás de otro formando grupos genéticos y reciben el nombre de genes PKS (Keller & Hohn 1997).

Dada la limitada diversidad de caracteres que poseen los líquenes alectorioides, la quimiotaxonomía ha sido ampliamente utilizada en este grupo. Esta tiene importancia tanto para distinguir géneros (por ejemplo, *Alectoria* de *Bryoria*), como especies. Los líquenes alectorioides pueden sintetizar una amplia variedad de compuestos liquénicos, entre los que destacan para la presente tésis, la atranorina y cloratranorina, y los ácidos alectoriálico, barbatólico, connorestíctico, fumarprotocetrárico, girofórico, norestíctico, protocetrárico y psoromico. No son pocas las especies de *Bryoria* que han sido descritas basándose en la composición de sus extrolitos. Algunas especies, como *Bryoria mariensis*, en la que predomina el ácido norestíctico, van acompañadas de una morfología peculiar (Fryday &

Øvstedal 2012). Sin embargo, otras especies, como *Bryoria salazinica*, que contiene ácido salazínico, fue descrita solo porque acumula esta sustancia (Brodo & Hawksworth 1977), siendo morfológicamente indistinguible de otras especies como *Bryoria pseudofuscescens*, por lo que un estudio molecular es recomendable para verificar la validez de este taxon. Un caso geneticamente estudiado es el de *Bryoria fremontii*, que puede presentar o no el tóxico ácido vulpínico en soralios y apotecios, y *Bryoria tortuosa*, que siempre lo produce por todo el talo. Resultados moleculares (Velmala *et al.* 2009) han demostrado que ambos taxones son conespecíficos (sinónimos), descartando que tanto la presencia o ausencia como la localización del ácido vulpínico sean caracteres filogenéticamente informativos. Mientras que la composición en sustancias liquénicas ha sido útil en la taxonomía de algunas especies de alectorioides, para otras se ha revelado como un carácter taxonómicamente artificial, lo que impide generalizar su uso.

El Clado Alectorioide

Dentro de la familia *Parmeliaceae*, el Clado Alectorioide forma un linaje monofilético que incluye cinco géneros: *Alectoria*, *Bryocaulon*, *Bryoria*, *Nodobryoria* y *Pseudephebe* (Divakar *et al.* 2015; Fig. 6). El aspecto capilar de estos líquenes es compartido por otros géneros de origen evolutivo muy dispar, como *Usnea*, *Protousnea*, *Ramalina*, *Oropogon*, *Sulcaria* o *Coelopogon*. Brodo & Hawksworth (1977), en su revisión del género *Alectoria* y géneros afines, empezaron a asentar las carácterísticas básicas del Clado Alectorioide, basándose en caracteres morfológicos, químicos y biogeográficos. Segregan el género *Bryoria* de *Alectoria* y consideran a *Pseudephebe*, *Oropogon* y *Sulcaria* como géneros afines. En aquel momento, *Bryocaulon* se consideraba un género más relacionado con otros grupos de parmeliáceos (Kärnefelt 1986), y *Nodobryoria* no se segregaría de la sección *Subdivergentes* de *Bryoria* hasta 1995 (Common 1995).

Aunque ya a mediados de los años 90 se habían descrito todos los géneros conocidos de líquenes alectorioides, tanto la relación entre ellos, como la familia en la que se ubicaban era dependiente de los carácteres utilizados por el autor del estudio, hasta que en 1999 los datos genéticos mostraron que pertenecían a la familia *Parmeliaceae* (Mattsson & Wedin 1999). No fue hasta el año 2007 cuando se empieza a hablar de líquenes alectorioides desde un punto de vista filogenético ya que entonces *Alectoria*, *Pseudephebe* y *Sulcaria* se revelan

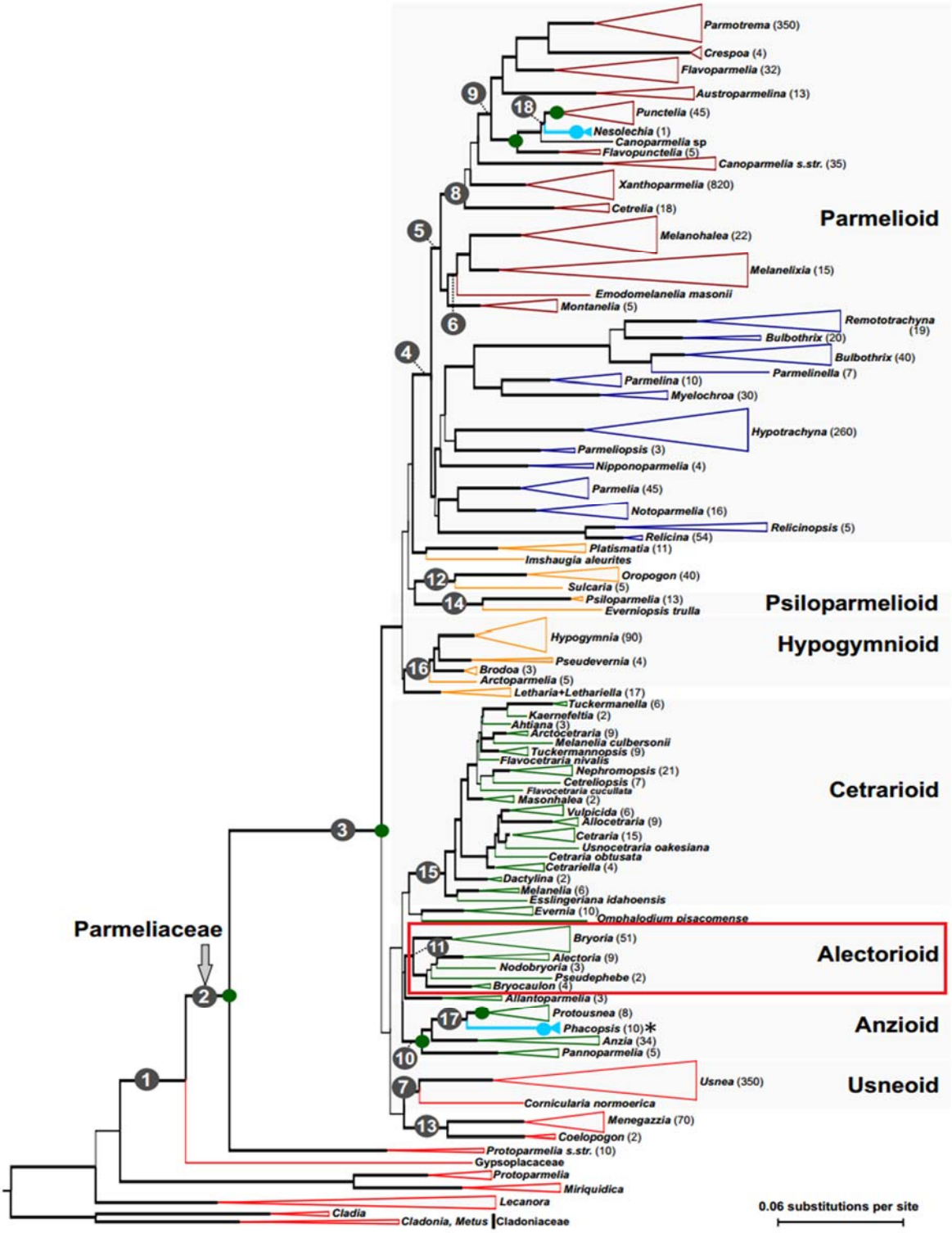


Fig. 6. Árbol filogenético de la familia *Parmeliaceae* indicando con un cuadro rojo la posición del Clado Alectorioide (imagen adaptada de la Fig. 1 en Divakar *et al.* 2015).

como un linaje monofilético (Crespo *et al.* 2007). No obstante, *Bryoria* y *Oropogon*, tenían todavía un parentesco incierto. En 2009 se describió el género *Gowardia* para acomodar a dos *Alectoria* terrícolas de coloración oscura (Halonen *et al.* 2009). La descripción de este

género se sustentaba principalmente en la publicación de un árbol filogenético en el cual *Alectoria* y *Gowardia* aparecían como dos linajes independientes. Posteriores trabajos que incluían más muestras y nuevos marcadores moleculares (Crespo *et al.* 2010; Thell *et al.* 2012), confirmaron que los géneros *Alectoria*, *Bryoria* y *Pseudephebe* formaban un clado monofilético, aunque *Sulcaria* también se situaban dentro de ellos. La aparición de *Alectoria* y *Gowardia* como linajes hermanos, hizo que este último nombre cayera en desuso. Finalmente, Divakar *et al.* (2015) revela que *Oropogon* y *Sulcaria*, generalmente tratados como líquenes alectorioides, no pertenecen a este clado, quedando reducido a los cinco géneros actualmente reconocidos.

El Clado Alectorioide incluye taxones morfológicamente poco variables, pero la disposición de las células del córtex, la coloración de las esporas y su composición en extrolitos, caracterizan a cada uno de los géneros. *Bryoria* es el género más aislado del clado, mientras que *Pseudephebe*, *Nodobryoria* y *Alectoria* tienen una relación incierta entre ellos. Se estima que el Clado Alectorioide apareció hace alrededor de 55 millones de años, no mucho después de los eventos que provocaron la extinción de los dinosaurios (Divakar *et al.* 2015).

El género *Bryoria*, con alrededor de 80 especies, es con diferencia el más numeroso y el único en el que se han reconocido secciones filogenéticas infragenéricas (Myllys *et al.* 2011a). La Tabla 1 muestra las cinco secciones aceptadas junto con algunos caracteres que permiten su identificación. La sección *Implexae*, en la cual se centra esta tesis, es taxonómicamente la más compleja, debido principalmente a la variabilidad fenotípica de las especies y a los pocos caracteres informativos que estas presentan. Por otra parte, *Pseudephebe*, con solo dos especies reconocidas, es el género más reducido.

Características de los líquenes alectorioides

Como ya hemos comentado, el Clado Alectorioide es un linaje monofilético de líquenes morfológicamente poco variables, con las estructuras típicas que aparecen en la gran mayoría de macrolíquenes fruticulosos. A continuación, se describen las principales características anatómicas, químicas y ecológicas que se pueden presentar en los géneros y especies de este grupo y especialmente en *Bryoria* y *Pseudephebe*.

Hábitat

Los líquenes alectorioides crecen principalmente en ambientes fríos y templados de todo el mundo, desde el ecuador hasta los polos. Generalmente están ligados a ambientes más o menos húmedos, muchas veces con frecuentes nieblas. Su principal zona de distribución es el área circumboreal que comprende el norte de América, Europa y Asia.

En menor medida aparecen en las zonas montañosas y templadas, escaseando cada vez más hacia latitudes ecuatoriales. En el Hemisferio Sur, vuelven a cobrar algo de importancia apareciendo, de manera menos frecuente que en el Hemisferio norte, en un área que podíamos denominar circumantártica (sur de Sudamérica, África y Oceanía). Se han llegado a encontrar en la Antártida continental (Green *et al.* 2011). Aunque generalmente son líquenes poco frecuentes, en algunas zonas de Canadá o de la Península Escandinava pueden ser abundantes y destacar, al menos visualmente, en el ecosistema (Fig. 7). Los puntos de mayor diversidad de líquenes alectorioides se encuentra en las zonas oceánicas del oeste de Norteamérica (Brodo & Hawksworth 1977), aunque parecen quedar todavía muchas especies por describir, por ejemplo, en Asia (Wang *et al.* 2017) y el Hemisferio Sur (Boluda *et al.* 2015). Cuando aparecen en las regiones tropicales, quedan restringidos a las altas montañas, llegando a superar los 4.400 metros de altitud (Swinscow & Krog 1988), mientras que, en áreas como la Península Antártica o Escandinava, pueden crecer al nivel del mar.

Fig. 7. Ambiente dominado por *Bryoria fremontii* y en menor proporción por *Bryoria fuscescens* s. l., típico de los bosques despejados y húmedos cercanos al círculo polar ártico. Noruega (fotos: C. G. Boluda).

Tabla 1. Secciones del género *Bryoria* y sus principales caracteres diferenciales. Solo las especies analizadas molecularmente se incluyen en la tabla. La especie tipo de cada sección aparece en negrita. (Adaptación de la Tabla 3 de Myllys *et al.* (2011a), con la adición de algunas especies posteriormente descritas).

Carácter	**Secciónes**				
	Americanae	***Bryoria***	***Divaricatae***	***Implexae***	***Tortuosae***
Química	Ácido fumarprotocetrárico	Generalmente ácido fumarprotocetrárico	A veces ácido fumarprotocetránico	Generalmente ácido fumarprotocetrárico	Ácido vulpínico
Pseudocifelas	Presentes	Presentes o ausentes	Presentes o ausentes	Presentes o ausentes	Presentes o ausentes
Soralios	Generalmente ausentes	Presentes o ausentes	Presentes o ausentes	Presentes o ausentes	Ocasionales
Hábito de las especies	Péndulo	Erecto, cespitoso, subpéndulo o péndulo	Erecto, cespitoso, raramente subpéndulo; bicolor	Subpéndulo o péndulo	Péndulo
Espínulas laterales o en las ramas	Presentes	Generalmente presentes	Presentes, constreñidas en la base	Ausentes	Ausentes
Taxones estudiados molecularmente	***Bryoria americana***	*B. alaskana* *B. carlottae* *B. divergescens* *B. fastigiata* *B. furcellata* *B. hengduanensis* *B. himalayana* *B. irwinii* *B. lactinea* *B. nadvornikiana* *B. nitidula* *B. perspinosa* *B. poeltii* *B. simplicior* ***B. trichodes***	*B. asiática* *B. barbata* ***B. bicolor*** *B. confusa* *B. fruticulosa* *B. indonésica* *B. nepalensis* *B. rigida* *B. ruwenzoriensis* *B. smithii* *B. tenuis* *B. variabilis* *B. wuii* *B. yunnana*	*B. capillaris* *B. friabilis* *B. fuscescens* *B. glabra* ***B. implexa*** *B. inctiva* *B. kockiana* *B. kuemmerleana* *B. lanestris* *B. pikei* *B. pseudofuscescens* *B. subcana* *B. vrangiana*	***B. fremontii***

La inmensa mayoría de especies son epífitas, aunque algunas, como *Bryoria nadvornikiana*, pueden ser saxícolas o epífitas, otras como *Pseudephebe minuscula* son saxícolas, y unas pocas especies, como *Bryocaulon hyperboreum*, son terrícolas. Las especies epífitas muestran una preferencia por las coníferas, aunque es difícil determinar si esto se debe a las características de su corteza, a que las coníferas crecen en ambientes adecuados para estos líquenes, o a una mezcla ambos factores (Hawksworth 1972). La corteza frecuentemente más lisa de los planifolios, unido a la poca luz presente generalmente en este tipo de bosques, pueden ser factores también a tener en cuenta. Las especies saxícolas suelen crecer sobre rocas silíceas, aunque en climas muy lluviosos, donde las sales se lavan con rapidez, pueden crecer sobre rocas calizas. Finalmente, las especies terrícolas son típicas de ambientes de tundra, creciendo entre el tapiz de líquenes, musgos y hierba baja del suelo (Thell & Kärnefelt 2011).

Los líquenes alectorioides no toman el agua del sustrato sobre el que viven, como hacen algunos foliáceos y crustáceos por simple capilaridad. Su hidratación viene dada principalmente a partir de la humedad atmosférica, ya sea del agua que se condensa entre sus lacinias, de la niebla o la lluvia. Su morfología capilar (que forman una especie de red tupida) les permite disponer de una gran superficie en relación con su masa, siendo muy eficientes a la hora de tomar el agua de una atmósfera con altos niveles de humedad. Sin embargo, cuando el aire es seco, esta característica también hace que se deshidraten rápidamente. Mientras que en áreas húmedas pueden aparecer en cualquier sitio del bosque, en zonas secas suelen quedar restringidas a barrancos y valles con pendiente, en los cuales el aire asciende, se enfría y produce nieblas frecuentemente. Suelen preferir como sustrato las ramas de los árboles, ya que generalmente están más iluminadas que los troncos, pero en lugares secos, quedan restringidas a los troncos. Al estar en contacto con la corteza, la cual mantiene la humedad mucho más tiempo que las ramas, el agua captada por los talos (en estos casos muy apoyados sobre el sustrato) tarda más en evaporarse. Además, pueden utilizar agua de escorrentía que absorben por capilaridad para mantenerse más tiempo activos (observaciones propias).

Es curioso que algunas especies de alectorioides, como *Pseudephebe minuscula* la más extremófila del grupo, sea capaz de crecer en latitudes tropicales como México y al mismo, tiempo a 84º sur, muy cerca del polo sur geográfico (Green *et al.* 2011). Podríamos pensar que las condiciones climáticas son extremadamente distintas, pero no hay que olvidar que los líquenes son organismos que pasan la mayor parte de su vida en latencia. Las condiciones ambientales que parecen influir más en su distribución, son las que están presentes en los momentos de actividad fisiológica. Así pues, la temperatura y humedad que activan al liquen en las zonas montañosas tropicales pueden ser las mismas que se alcanza

puntualmente en los micronichos de la Antártida continental. Además, esta especie es capaz de adaptar su morfología, pasando de fruticulosa a formar parches cási crustáceos, absorbiendo mejor el calor de la roca y evitando la pérdida de agua y temperatura. Mientras que las condiciones ambientales en momentos de actividad fisiológica pueden ser muy parecidas, la periodicidad y duración de estas no, provocando que las tasas de crecimiento en los diversos ambientes sean muy distintas (Sancho *et al.* 2007).

Hábito

Una característica común a todos los líquenes alectorioides es su biotipo fruticuloso y su aspecto capilar. No obstante, dependiendo de la longitud y rigidez de las lacinias, existen varios términos generalmente utilizados en liquenología para designar mejor a los distintos tipos de talos fruticulosos (Fig. 8). Los talos cespitosos se caracterizan por formar matas arbustivas, generalmente menos del doble de largas que anchas. Con frecuencia, desde la base surgen ramas robustas más o menos horizontales que forman ramas secundarias. Los talos erectos tienen aspecto de arbusto enano, con ramas principales que forman ramificaciones secundarias. Los talos subpéndulos constan de ramas más o menos endurecidas en la base, que pueden crecer hacia arriba, pero terminan volviéndose péndulas por su propio peso. Los talos péndulos son colgantes y están formados por lacinias que se disponen verticalmente hacia el suelo desde la base. Finalmente, las especies decumbentes tienen talos que crecen a lo largo del sustrato formando almohadillas o rosetas (Brodo & Hawksworth 1977; Fig. 8).

Un carácter importante en los líquenes alectorioides es su patrón de ramificación. Las ramificaciones, salvo excepciones, son dicótomas, es decir, se dividen de dos en dos. Pueden formar dos ramas iguales (isótomas) o diferentes (anisótomas), una principal y otra secundaria. Así pues, los tipos de ramificación más frecuentes son los isótomo-dicótomos y los anisótomo-dicótomos. Las especies erectas, por ejemplo, son siempre anisótomas. Entre las dos ramas formadas en una ramificación dicotómica existe un ángulo. Este es un carácter taxonómico importante para algunas especies, pudiendo ser agudo u obtuso y, además, afilado o redondeado. La presencia de pequeñas ramillas laterales en las ramas de último orden, que suelen recibir el nombre de espínulas, son otro carácter taxonómicamente informativo (Brodo & Hawksworth 1977).

Fig. 8. Ejemplos de hábitos en líquenes alectorioides. A, *Bryocaulon divergens* con talos postrados, Noruega. B, *Bryoria ruwenzoriensis*, una especie con ramas erectas, Tanzania. C, *Bryoria fuscescens*, una especie péndula, Portugal (fotos: C. G. Boluda).

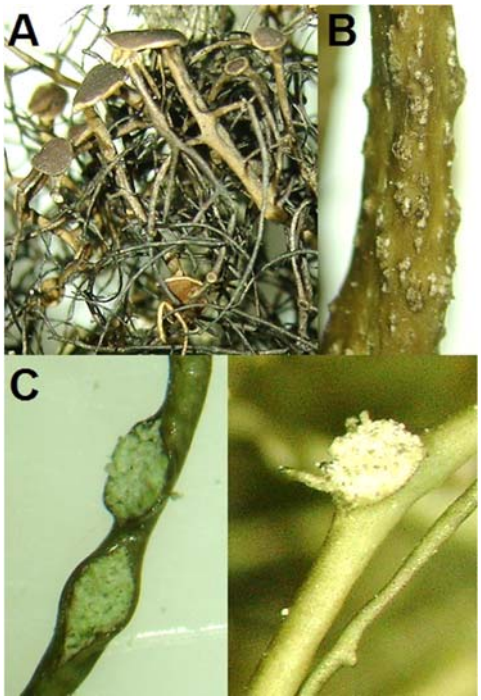


Fig. 9. A, Apotecios de *Nodobryoria abbreviata*. B, pseudocifelas en *Bryoria fremontii*. C, soralios fisurales (izquierda) y tuberculares (derecha) de *Bryoria fuscescens* (fotos: C. G. Boluda).

Estructuras de aireación

En los momentos de actividad fisiológica, mantener el talo bien aireado es importante, no solo para favorecer un buen intercambio gaseoso, sino también para minimizar que el dióxido de carbono se transforme en ácido carbónico, menos utilizable por las algas clorófitas. Las lacinias de los líquenes alectorioides son esencialmente cilindros, con pequeños huecos entre las hifas de la médula, que por capilaridad tenderían a llenarse de agua. Sin embargo, esto se evita gracias a sustancias hidrofóbicas, como pueden ser algunos metabolitos secundarios o proteínas como las hidrofobinas (Armaleo 1993; Huneck 1999). El interior medular hueco, además, ve favorecida su aireación mediante la formación de pseudocifelas, que son regiones de la superficie que carecen de córtex, comunicando la médula con el exterior (Fig. 9B).

Las pseudocifelas suelen tener mucha importancia taxonómica en los líquenes parmeliáceos, siendo útiles, por ejemplo, para diferenciar géneros (Thell & Moberg 2011). En los líquenes alectorioides, las pseudocifelas permiten distinguir algunos géneros y especies. Además, permiten descartar géneros parecidos, pero filogenéticamente alejados, como

Oropogon, que con frecuencia las tiene perforadas, o *Sulcaria*, con pseudocifelas espiraladas y muy desarrolladas. Las pseudocifelas son llamativas en el género *Alectoria*, donde generalmente sobresalen del talo. En el género *Bryoria* son variables, de blancas a concoloras con el talo, generalmente fusiformes, a veces con extrolitos que no aparecen en el resto del liquen, y en ocasiones inconspicuas. En algunas especies, como *Bryoria fuscescens*, las pseudocifelas se han descrito como estadíos iniciales de la formación de los soralios, debido a su reducido desarrollo (Myllys *et al* 2011b). En *Pseudephebe*, aunque en ocasiones pueden ser muy aparentes, pasaron desapercibidas cuando se describió el género. Sin embargo, un estudio detallado, muestra que ambos grupos presentan pseudocifelas, como se menciona en los capítulos uno y siete (Boluda *et al.* 2016).

Reproducción asexual

Como en otros líquenes, los alectorioides se reproducen principalmente de forma asexual. Dada su naturaleza, cualquier fragmento que contenga al micobionte y fotobionte puede servir de propágulo viable. Así pues, la fragmentación del talo es una forma común de reproducción para muchas de estas especies. El género *Nodobryoria* (Fig. 9A) suele tener talos rígidos y frágiles, que favorecen su ruptura por presión y, por tanto, su dispersión. Dentro del género *Bryoria*, se han descrito especies en las que la friabilidad de sus talos es un carácter taxonómicamente importante, como el caso de *Bryoria friabilis* o *Bryoria lanestris*, aunque como se verá más adelante, estas especies han terminado siendo sinonimizadas en el transcurso de la presente tesis (Brodo & Hawksworth 1977; Myllys *et al.* 2011b).

Los isidios son estructuras de reproducción asexual típicas de muchos líquenes. Se caracterizan por ser pequeñas protuberancias corticales, generalmente cilíndricas o coraloides, fácilmente separables del talo, que contienen pequeñas porciones de médula (incluyendo algas) protegidas por un córtex. Se suelen distinguir de las espínulas porque estas últimas no tienen la base constreñida ni se desprenden fácilmente. Los líquenes alectorioides carecen de verdaderos isidios, pero unas pocas especies presentan espínulas isidioides. Estas estructuras caracterizan por ejemplo a *Alectoria imshaugii*, donde aparecen en las pseudocifelas, y a *Bryoria furcellata*, en la cual crecen en los soralios. La facilidad con la que se desprenden del talo se debe a la zona en la que crecen, en la cual, la médula, compuesta por hifas laxas, asoma al exterior. Es por esto que no pueden considerarse verdaderos isidios (Brodo & Hawksworth 1977).

Los soredios son probablemente las estructuras de reproducción asexual más utilizadas en los líquenes. Son gránulos microscópicos con aglomeraciones de hifas y algas producidas en zonas decorticadas, llamadas soralios. Los soralios se desarrollan en muchas de las especies de líquenes alectorioides y siempre aparecen como estructuras delimitadas y

discretas (Fig. 9C). En *Bryoria* se reconocen dos tipos principales de soralios, los fisurales (rimiformes), generalmente elípticos, planos o cóncavos, originados a partir de una fisura del córtex, y los tuberculares, generalmente redondeados, o irregulares, sobresalientes y con frecuencia con un reborde cortical (Fig. 9C). Tanto su presencia o ausencia como sus características morfológicas tienen importancia taxonómica (Velmala *et al.* 2014; Barreno & Rico 1984).

Los picnidios, estructuras especializadas en la formación de diminutas esporas asexuales (conidios), suelen ser raros o están ausentes en la mayoría de las especies. Son especialmente frecuentes en el género *Pseudephebe*. Dado su pequeño tamaño, se espera que su capacidad de dispersión sea elevada, no obstante, al carecer del fotobionte, su éxito reproductivo se ve limitado. A día de hoy y en el grupo que estudiamos, se desconoce si los conidios son solo propágulos para la reproducción asexual o funcionan también como espermacios del micobionte, participando por tanto en la reproducción sexual (Honegger 1984).

Reproducción sexual

Como se comentaba anteriormente, los micobiontes de los líquenes son polifiléticos y en muchos de sus linajes se desconocen los ciclos de vida completos. El apotecio es el típico órgano de reproducción sexual en los líquenes alectorioides. Mientras que su desarrollo y presencia es frecuente en algunas especies, sobre todo en aquellas que tienen talos cespitosos o erectos, es muy raro en otras o incluso nunca se han podido observar. Tanto las características de sus esporas, como las estructurales del propio apotecio, son de gran valor taxonómico para la mayoría de los líquenes. La relativa ausencia de apotecios en los líquenes alectorioides ha favorecido que no adquieran una importancia taxonómica destacable. Las ascósporas de los líquenes alectorioides se caracterizan por ser simples, lo que permite distinguir a este grupo de géneros como *Oropogon* o *Sulcaria*, con esporas monoseptadas a murales. Por norma general los ascos contienen 8 esporas hialinas, aunque en el género *Alectoria* este número varía de 2 a 4, son pardas y más grandes que en el resto de géneros alectorioides. Los apotecios tienen las características típicas de los líquenes parmeliáceos, con excípulo talino, persistente e incurvado en *Alectoria* y excluido al madurar en el resto de géneros (Brodo & Hawksworth 1977).

La producción de apotecios en algunos taxones va ligado a ciertas áreas geográficas que consideramos por ello óptimas para dicha especie, como es el caso de *Bryoria* sección *Implexae*. Sin embargo, su desarrollo no siempre conlleva a la formación de ascosporas, ya que, en este grupo raramente pueden encontrarse ascos fértiles.

Anatomía de los tejidos vegetativos

Todos los líquenes alectorioides tienen una morfología y anatomía bastante característica. Los especímenes jóvenes suelen tener un disco de adhesión al sustrato, aunque frecuentemente este desaparece y el ejemplar se sujeta mediante la fricción que le proporcionan sus ramas. En ocasiones, pueden aparecer discos de adhesión secundarios, sobre todo en el género *Pseudephebe*.

Las lacinias son cilíndricas y con una disposición radial de sus estratos. El córtex suele ser ± grueso y la médula muy laxa o, en ocasiones, apenas desarrollada. La integridad estructural de los talos viene dada por el córtex, que actua a modo de exoesqueleto, y está formado básicamente por hifas dispuestas longitudinalmente (periclinales), de paredes más o menos gruesas que forman una matriz mucilaginosa que las mantiene cohesionadas. El córtex es, por tanto, prosoplectenquimático (Barreno & Rico 1984). El grosor de las paredes mucilaginizadas de las hifas del cortex es variable en los géneros y especies alectorioides. En algunas especies, las hifas son mesodermas, mientras que en otras son paquidermas, es decir el lumen es estrecho y la pared mucilaginizada muy gruesa, llegando a ser el componente estructural mayoritario del córtex (Brodo & Hawksworth 1977; Barreno & Rico 1984). Así, *Bryoria friabilis* y *B. lanestris*, con lacinias frágiles y fácilmente fragmentables, desarrollan un córtex con hifas ± mesodermas, mientras que *B. glabra*, producen un córtex muy mucilaginoso de hifas claramente paquidermas, dando lugar a lacinias más resistentes y difíciles de fragmentar. Mientras que las partes más internas del córtex están principalmente compuestas por hifas dispuestas periclinalmente, la fila más externa de hifas es variable, suele estar pigmentada y es taxonómicamente importante. La forma de las células superficiales, así como su disposición, permite distinguir a los líquenes alectorioides de otros parecidos, como *Oropogon*, *Sulcaria* o *Coelopogon*. Además, dentro de los líquenes alectorioides, esta característica sirve para diferenciar géneros. Por ejemplo, en *Nodobryoria*, estas hifas son irregulares, cortas y tortuosas, en *Pseudephebe* la fila externa de células se dispone en un prosoplecténquima de hifas en red, mientras que las inferiores son claramente periclinales. En *Bryoria*, simplemente se disponen periclinalmente. Por encima del córtex algunas especies acumulan sustancias o pigmentos, formando un epicortex (Barreno & Rico 1984). En *Bryoria* el grosor del epicortex en los diferentes taxones puede ser muy variable y de probable interés taxonómico. Aunque en los líquenes alectorioides aparecen polisacáridos como el liquenano e isoliquenano (Common 1991), desconocemos si otras sustancias no glucosídicas forman parte de esta capa (Brodo & Hawksworth 1977).

La médula en los líquenes alectorioides es muy laxa, de tipo aracnoide, formada por hifas de 3.5–5 µm de diámetro, generalmente con la pared lisa, aunque en *Alectoria* y en raras

ocasiones en *Bryoria*, puede estar ornamentada. En las especies con la médula más densa, como *Alectoria vancouverensis*, pueden aparecer haces de hifas medulares dispuestas de forma similar al córtex, uniéndose parcialmente a este en algunos puntos. Las células del fotobionte se disponen principalmente en la cara interna del córtex, aunque algunas algas pueden aparecer en las regiones centrales de la médula.

Justificación y objetivos

***Bryoria smithii* y *B. bicolor* creciendo entremezcladas.**
España, Asturias, (foto: C. G. Boluda).

Justificación y objetivos

Justificación

Los estudios filogenéticos realizados en la familia *Parmeliaceae*, han mostrado frecuencia fuertes discrepancias entre el concepto de especie morfológico (especies tradicionalmente aceptadas) y filogenético (Molina *et al.* 2011a; 2011b; Nuñez-Zapata *et al.* 2011; Altermann *et al.* 2014; Saag *et al.* 2014; Del-Prado *et al.* 2016). Los líquenes del Clado Alectorioide, debido a los pocos caracteres fenotípicos que presentan, parecen susceptibles a este problema. Algunas especies son tan variables que se reconoce cierto solapamiento entre ellas, como ocurre entre *Pseudephebe minuscula* y *P. pubescens* o entre *Alectoria imshaugii*, *A. sarmentosa*, *A. sorediosa*, *A. vancouverensis* y *A. vexillifera*. El solapamiento fenotípico parece especialmente acusado en *Bryoria* sect. *Implexae*, donde se pueden encontrar dos grandes grupos fenotípicos, el de *Bryoria austromontana*, *B. chalybeiformis*, *B. friabilis*, *B. fuscescens*, *B. glabra*, *B. implexa*, *B. inactiva*, *B. kockiana*, *B. kuemmerleana*, *B. lanestris*, *B. pseudofuscescens*, *B. salazinica* y *B. vrangiana*, y el complejo de *Bryoria capillaris* y *B. pikei*.

Un reciente estudio filogenético en el complejo de *Alectoria sarmentosa* (McMullin *et al.* 2016) muestra que las secuencias de marcadores estándar de ADN no tienen resolución suficiente para formar linajes, quedando en duda si las especies morfológicas pueden ser validadas filogenéticamente. En el caso de *Bryoria* sect. *Implexae*, el grupo mejor estudiado de entre todos los del Clado Aectorioide, ocurre algo similar. Aparecen como monofiléticos cuatro grandes linajes muy emparentados (Velmala *et al.* 2014; Fig 10). Por una parte *Bryoria glabra*, que forma un clado propio, mientras que, por otra, aparecen tres grandes clados, cada uno con varios de taxones. El primero incluye a *B. kockiana* y una *Bryoria* indeterminada. El segundo está compuesto por individuos exclusivamente norteamericanos. Finalmente el tercero consta de individuos ampliamente distribuidos. Sin embargo, la resolución dentro de estos grupos es tan baja y con tal cantidad de secuencias clonales, que no permite distinguir si las especies morfológicas merecen reconocimiento filogenético. Sobre la base de estos resultados, y al mismo tiempo que la tesis tenía lugar, algunas especies de *Bryoria* se sinonimizaron, como *B. chalybeiformis*, *B. lanestris* y *B. subcana* (Velmala *et al.* 2014). No obstante, el resto de especies de la sección *Implexae* permanecen bajo el témino de aceptadas, principalmente debido a que presentan caracteres morfológicos o químicos distintivos (Velmala *et al.* 2014; Tabla 2).

Las especies de *Bryoria* sect. *Implexae*, debido a las condiciones ambientales que necesitan para vivir, han sido utilizadas históricamente como indicadores de la buena calidad del bosque (Esseen *et al.* 1996; Liira & Sepp 2009). Se ven fácilmente afectadas por cambios estructurales de su hábitat, por la contaminación atmosférica o por las actividades humanas (Thor 1997; Walker *et al.* 2006). Mientras que en el Norte de Europa estas especie no suelen ser raras, en el Centro y Sur se encuentran en regresión (como indican las citas de herbario), quedado restringidas a las montañas elevadas y húmedas, en hábitats muy susceptibles al calentamiento global. Para entender mejor como las características ambientales actúan modelando la riqueza genética y fenotípica de las poblaciones de estos líquenes, así como sus capacidades de dispersión y adaptabilidad frente a cambios climáticos, en esta tesis se realiza un estudio poblacional en uno de los linajes de *Bryoria* sect. *Implexae* utilizando especímenes de Europa y Norte de África.

Tabla 2. Sustancias químicas diagnóstico que junto con carácteres adicionales permiten distinguir las especies de *Bryoria* sect. *Implexae*. Los carácteres adicionales incluyen la coloración del talo, los ángulos de ramificación, las características de los soralios y pseudocifelas y la distribución.

Especie	Sustancia diagnóstico
Bryoria austromontana	Ácido fumarprotocetrárico
B. capillaris	Ácido barbatólico
B. friabilis	Ácido girofórico
B. fuscescens	Ácido fumarprotocetrárico
B. glabra	Ácido fumarprotocetrárico
B. implexa	Ácido psorómico
B. inactiva	Sin sustancias
B. kockiana	Ácido psorómico
B. pikei	Ácido barbatólico
B. pseudofuscescens	Ácido norestíctico
B. salazinica	Ácido salazínico
B. vrangiana	Ácido fumarprotocetrárico

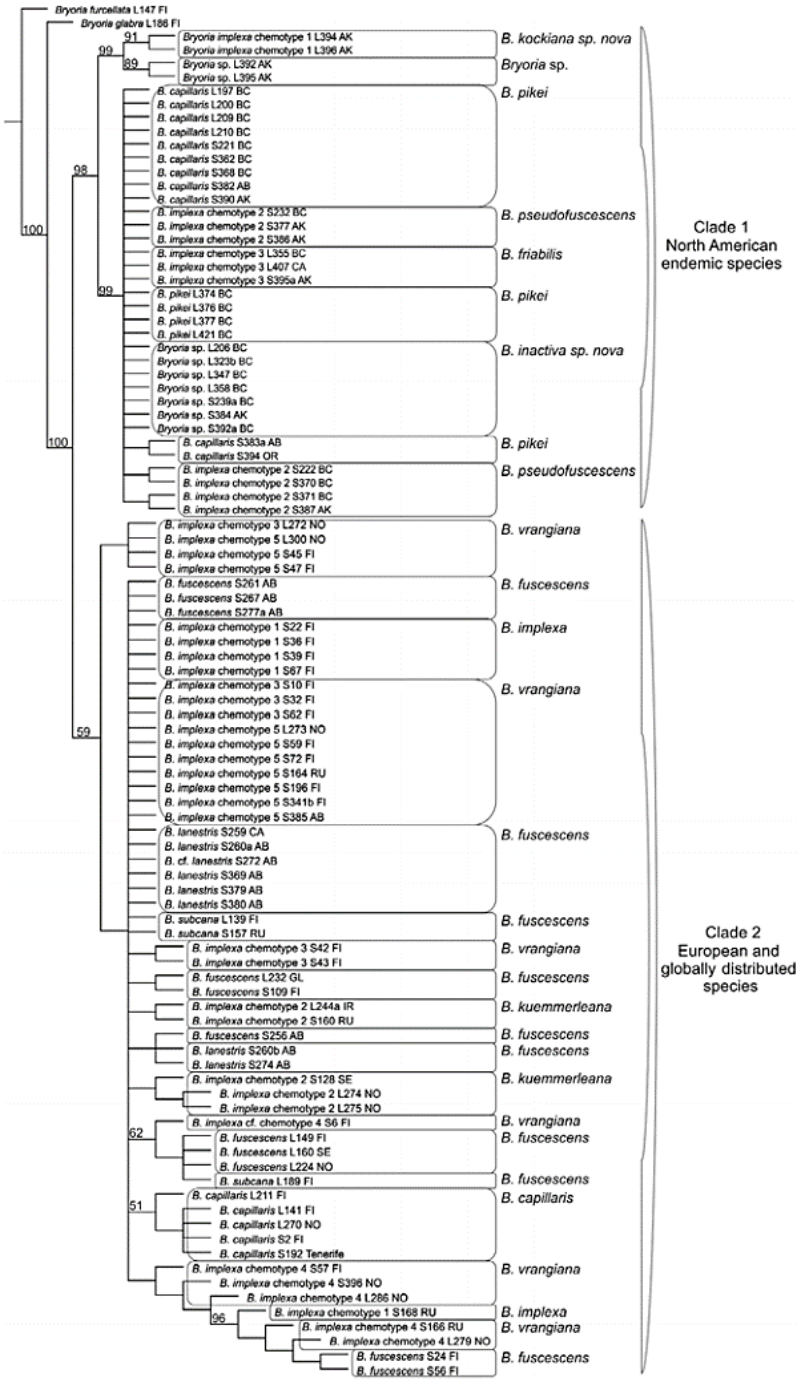


Fig. 10. Reconstrucción filogenética tomada de Velmala *et al.* (2014: Fig. 3), que utiliza una combinación de marcadores moleculares (ITS, IGS y GAPDH), datos químicos, morfológicos y corológicos.

En esta tesis se pretende averiguar, como objetivo amplio, a qué podemos calificar como especie, es decir trataremos de resolver el concepto de especie, en dos linajes de líquenes (géneros) del Clado Alectorioide que parecen haber sufrido procesos de especiación opuestos: *Pseudephebe* y *Bryoria* sect. *Implexae*. También se procederá a aclarar la posición

filogenética de *Bryoria mariensis* y de muestras de *Bryoria* recolectadas en el Hemisferio Sur (Chile), así como estudiar la utilidad del uso de los extrolitos como caracteres diferenciales en la taxonomía de estos grupos. También se ha contemplado la realización de un estudio poblacional en *Bryoria* sect. *Implexae* en Europa y el Norte de África, para estudiar su diversidad genética y facilitar y apoyar la valoración del concepto de especie en este grupo.

Objetivos

Los objetivos específicos establecidos en esta tesis doctoral son:

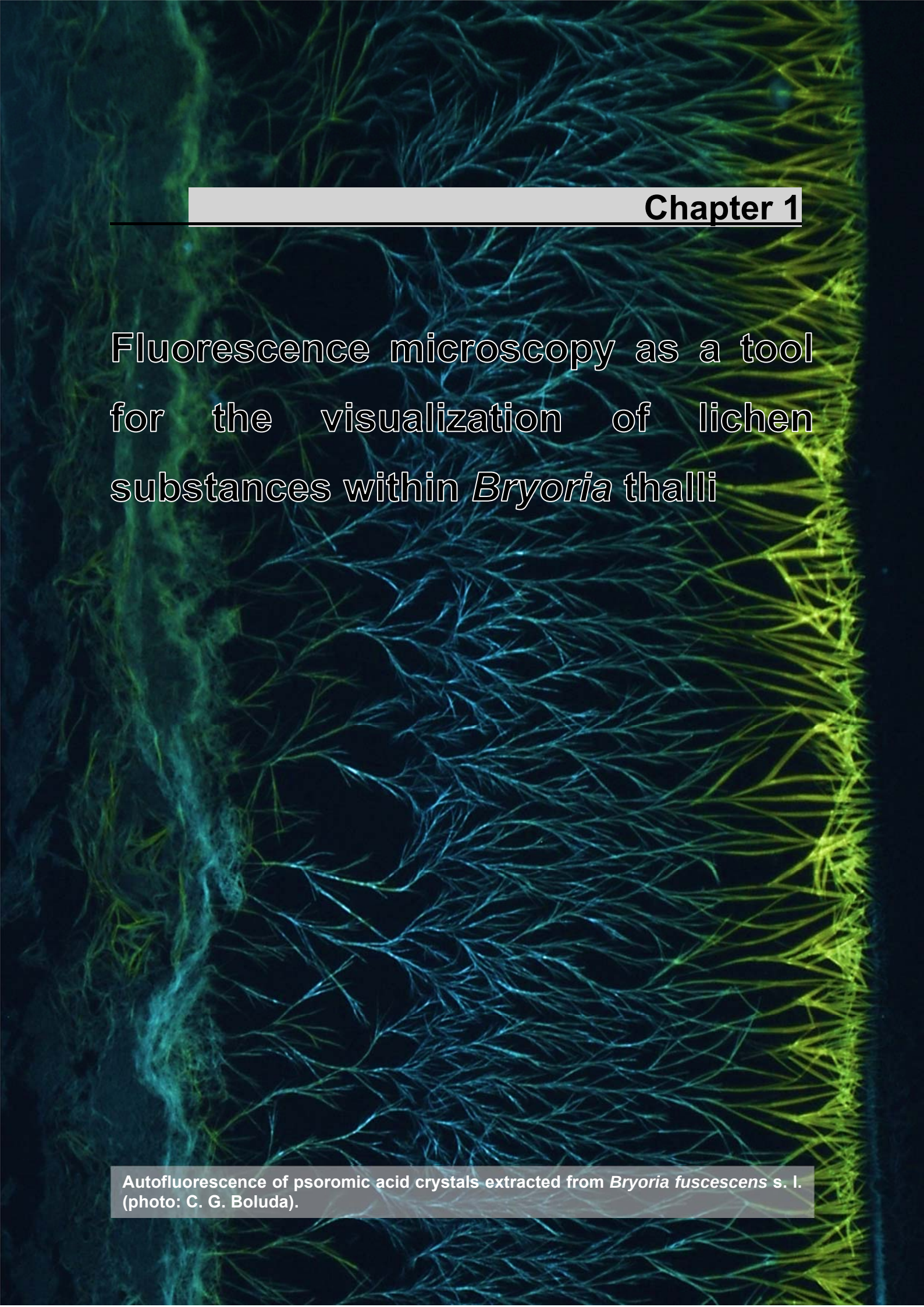
- Testar la utilidad de la autofluorescencia a nivel microscópico para detectar la ubicación de extrolitos u otras sustancias acumuladas (Capítulo 1).
- Averiguar si los extrolitos producidos por los líquenes de *Bryoria* sect. *Implexae* se distribuyen por todo el talo o se acumulan en regiones concretas (Capítulos 1 y 2).
- Explorar la relación entre composición en extrolitos y parentesco genético en individuos geográficamente cercanos y morfológicamente similares (Capítulo 2).
- Obtener marcadores genéticos de alta variabilidad (microsatélites y secuencias intergénicas altamente variables) que permitan realizar un estudio poblacional y filogenético en *Bryoria* sect. *Implexae* (Capítulos 3 y 5).
- Resolver el concepto de especie en *Bryoria* sect. *Implexae*, mediante una aproximación integrativa y utilizando marcadores de ADN altamente variables (Capítulo 4).
- Realizar un estudio poblacional y filogeográfico de *Bryoria fuscescens* agg. en Europa y el Norte de África (Capítulo 5).
- Averiguar las posibles causas que pueden provocar la alta variabilidad fenotípica en *Bryoria fuscescens* agg. (Capítulo 5).
- Comprobar si el hongo liquenícola *Raesaenenia huuskonenii* tiene preferencia por ciertos fenotipos/taxones de *Bryoria* sect. *Implexae*. (Capítulo 5).
- Determinar filogenéticamente la especie a la que pertenecen ejemplares de *Bryoria* sp. recolectados en Chile (Capítulo 6).
- Analizar la adscripción genérica de *Bryoria mariensis*, un taxón con características intermedias entre varios géneros (Capítulo 7).
- Establecer el concepto de especie en el género *Pseudephebe* (Capítulo 7).

Capítulos/Chapters

Bryoria fuscescens s. l., Portugal (foto: C. G. Boluda).

Chapter 1

Fluorescence microscopy as a tool for the visualization of lichen substances within *Bryoria* thalli



Autofluorescence of psoromic acid crystals extracted from *Bryoria fuscescens* s. l. (photo: C. G. Boluda).

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Introduction

In some species of *Bryoria* (*Parmeliaceae*), including *Bryoria bicolor* (Ehrh.) Brodo & D. Hawksw. and *B. fuscescens* (Gyeln.) Brodo & D. Hawksw., thallus reactions with spot tests are patchy, and phrases such as ‘‘Pd+ bright red at least in parts’’ have been used in descriptions for many years (e.g. Hawksworth 1972). It has recently been discovered that the extracellular lichen substances (‘extrolites’) in individual thalli of some species in the genus are more varied than hitherto assumed (Hawksworth *et al*. 2011; Myllys *et al*. 2011). We hypothesized that this variation in extrolites and the patchiness phenomenon could be due to one or more of several factors, including: 1) chemosyndromic variation; 2) variations in concentration and the sensitivity of detection methods; 3) differences between basal, median and apical regions of the thallus; or 4) the localization of particular compounds in particular anatomical features, such as pseudocyphellae and soralia. As the localization of compounds is known to occur in at least one species of the genus, *viz*. the yellow pigment vulpinic acid in the soralia of *Bryoria fremontii* (Brodo & Hawksworth 1977), we decided to explore the last possibility first.

Long-wave ultra-violet (UV) fluorescence (at 350 nm) has proved a valuable tool in the separation of thalli of similar crustose and macrolichens, and is also used routinely in the examination of thin-layer chromatographic (TLC) plates; xanthones fluoresce shades of yellow, orange and red, while depsides and depsidones generally fluoresce blue to white or shades of grey, although atranorin gives a yellow hue (Orange 2010). In addition, fluorescence microscopy has been used to explore the location of lichen products in sections of a range of macrolichens (Kauppi & Verseghy-Patay 1990). We therefore decided to explore whether fluorescence microscopy could be used to determine the localization of extrolites in whole lichen thalli, as a supplement to reagent tests, especially as material subjected to Pd reactions has to be discarded. In addition, we wished to determine whether fluorescence would disclose sites: 1) not revealed by reagent tests, that is sites where compounds were present in concentrations too low to yield visible spot test reactions; and 2) which fluoresced in different colours, suggesting the presence of more than one compound.

Materials and Methods

In this investigation, we used specimens from populations of *Byoria* sect. *Implexae* (Myllys *et al.* 2011) collected in several European countries, but especially in the central mountains of Spain (deposited in MAF-Lich.). We examined transverse and longitudinal sections cut by hand, and also thallus portions, mounted in water. Spot tests and TLC were performed using standard methods and solvents A, B, C, and G (Orange *et al*. 2010). For auto-fluorescence we used a Nikon microscope: D-LF epi-fluorescence module coupled to an Eclipse-80i, with bright field and DIC, and connected to a DS-Fi1 camera and DS-C2 control unit. Two filter blocks were used: Nikon UV-2A (Ex 330–380 nm, DM 400, BA 420) and Nikon B-2A (Ex 450–490 nm, DM 505, BA 520).

Results and Discussion

The most striking fluorescence was the red under the UV-2A and B-2A blocks, attributable to the chlorophyll of the included *Trebouxia* cells. This fluorescence is widely used in plant and lichen physiology as an indicator of the condition of chloroplasts and algal cells (Maxwell & Johnson 2000; Jensen 2002; Jensen & Kricke 2002). Red fluorescence, therefore, disappears in old collections (e.g. MAF-Lich. 584 from Madrid collected in 1973, MAF-Lich. 586 from Navarra collected in 1984, and MAF-Lich. 4248 from British Columbia collected in 1994). We also found that if fresh thalli were exposed to the UV sources for just 15 min, the red fluorescence becomes white, indicating chlorophyll damage. Furthermore, some regions of thallus branches were whitish blue in the algal layer instead of red, which suggests algae in some parts of thalli may be dying. Interestingly, in low-magnification bright-field microscopy, the same algae are not significantly damaged.

A bluish green fluorescence under UV-2A was evident in the granular outer layer (Fig. 1B). This consisted of small granules when sparse, but a cracked film when abundant. This variation in the surface features of *Byoria* sect. *Implexae* specimens is evident in scanning electron micrographs (SEM; Hawksworth 1969: Figs 1a–d, 2a–c). The granules are extracellular and develop at a short distance behind the growing tips, and can be removed by treatment with KOH (K) and the lipase/protease enzymes in biological washing powders (Greenhalgh & Whitfield 1987). Rikkinen (1995) postulated that these granules might have a lightscattering function. *Bryoria fuscescens* can have an almost black to an almost white cortex. Under fluorescence microscopy, we found that in dark thalli this substance was hardly evident (Fig. 1A left), while in whitish thalli the granules covered the entire cortex which fluoresced

bluish green (Fig. 1A right). Our material of *Bryoria capillaris* (Ach.) Brodo & D. Hawksw., with a whitish grey thallus, always fluoresced intensely bluish green in the granular outer layer (Fig. 1E). This granular fluorescent substance was more abundant in older parts of the thalli, and rarer or absent in the tips, an observation consistent with those of Greenhalgh & Whitfield (1987). Protocetraric/fumarprotocetraric, norstictic/connorstictic, and psoromic acids gave variations of a whitish blue to greenish blue sequence of fluorescence colours, not clearly differentiated. However, the autofluorescence did serve to demonstrate extrolite distribution in the thallus using three consecutive portions of one branch of a specimen with only one TLC-detected extrolite: 1) TLC, to identify the unique lichen substance; 2) autofluorescence (UV-2A) before and after a K spot test; and 3) extrolite removal with an acetone bath (2 h at 20 ºC), then autofluoresence (UV-2A) before and after a K spot test. An example is specimens of *Bryoria fuscescens* with norstictic acid confined to soralia or pseudocyphellae with whitish blue fluorescence (Fig. 1D top). Adding K after fluorescence observation, the typical red acicular crystals formed in these laciniae areas, while other parts of the thallus gave no reaction (Fig. 1D bottom). After removing norstictic acid with acetone and adding K, the red crystals of the reaction were not observed by bright-field microscopy, indicating the acid had been removed. However, when that sample was examined under fluorescence, a less intense whitish blue colour remained, suggesting that either a little of the acid remained or there was another fluorescent substance present not soluble in acetone. Pseudocyphellae and soralia fluoresced the brightest (Fig. 1C & F). Further tests using the described combined method showed that this fluorescence was not exclusively attributable to substances detectable by TLC. This phenomenon was not restricted to *Bryoria* sect. *Implexae*. We also studied specimens of *Bryoria bicolor*, which accumulate fumarprotocetraric acid in the thallus but not in the pseudocyphellae (Pd-). Under the fluorescence microscope, the pseudocyphellae fluoresced brightly with the same colour as those of *B. fuscescens*. This suggests that this localized fluorescence in the absence of the acids is attributable to different substances. We speculated if hydrophobins could be the cause, peptide-containing proteins that self-assemble on the surfaces of hyphae, but these are not expected to fluoresce, unless labelled (Wang *et al*. 2002). The nature of this fluorescent material remains obscure and merits detailed study.

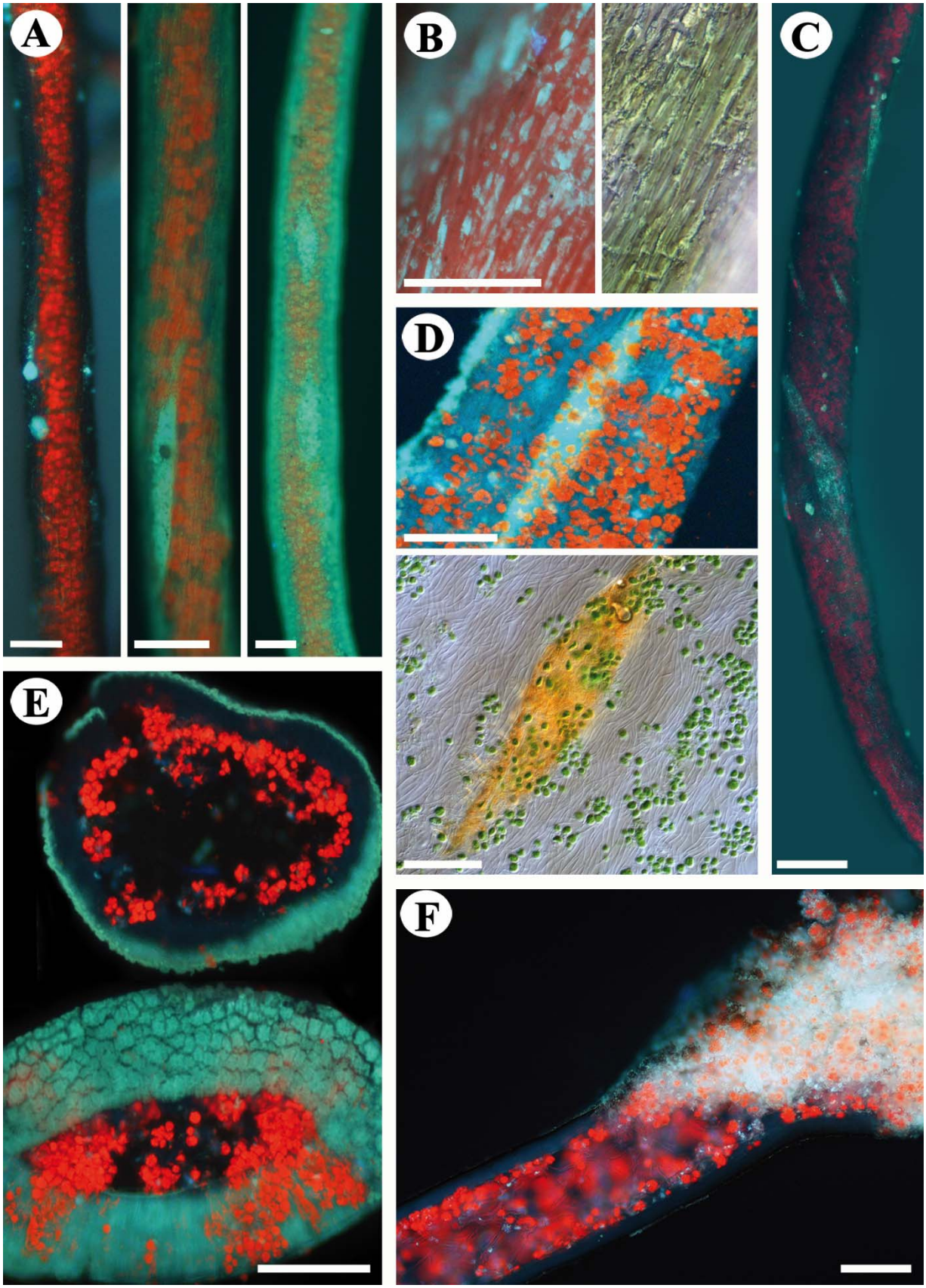
A
B
C
D
E
F

Fig. 1. *Bryoria* auto-fluorescence, using UV-2A cube (except in B right and D down) and DIC. Red fluorescent colours are produced by *Trebouxia* chlorophyll. **A**, *Bryoria fuscescens*, granular outer layer: left, black specimen (Spain, Canary Islands, Gran Canaria, MAF-Lich. 18859); centre, brown specimen (Spain, Asturias, Caso, MAF-Lich. 18860); right, pale grey specimen (Spain, Canary Islands, Tenerife, MAF-Lich. 18861). Fusiform brightest areas correspond to an inconspicuous pseudocyphella and circular bright areas to an incipient soralium; **B**, *B. fuscescens*, dark olive specimen granular outer layer with (left) and without fluorescence (Spain, Madrid, MAF-Lich. 18862); **C**, *B. fuscescens*, dark grey lacinia with bluish fluorescent pseudocyphellae (Spain, Segovia, MAF-Lich. 18863); **D**, *B. fuscescens* (Spain, Madrid, MAF-Lich. 18862), dark olive specimen with inconspicuous pseudocyphellae, containing norstictic acid as unique lichen compound (TLC): up, with epi-fluorescence; down, acicular crystals after adding K, without fluorescence; **E**, *B. capillaris*, cross-sections of a pale grey specimen, showing the granular outer layer (Spain, Segovia, MAF-Lich. 18865); **F**, *B. fuscescens*, longitudinal lacinia section of a dark grey specimen with brightly fluorescent soralium (Spain, Segovia, MAF-Lich. 18864). Scales = 100 µm.

←

In summary, this preliminary investigation into the possibilities of the use of fluorescence microscopy for the localization of extrolites in *Bryoria* thalli, indicates that it has the potential to precisely demonstrate heterogeneous deposition sites. However, at least with the method and UV-blocks used and the compounds accumulated in *Bryoria*, the colours produced were not sufficiently distinctive to enable particular lichen products to be differentiated by eye, though it is probable that they could be separated spectrophotometrically. Furthermore, the method evidently has to be used with caution as we discovered areas of localized fluorescence where lichen acids were absent or had been eluted, notably granules on the surface, soralia, and pseudocyphellae.

Acknowledgements

This contribution was prepared with support from the Spanish Ministerio de Economía y Competitividad project CGL2011-25003.

Additional Pictures

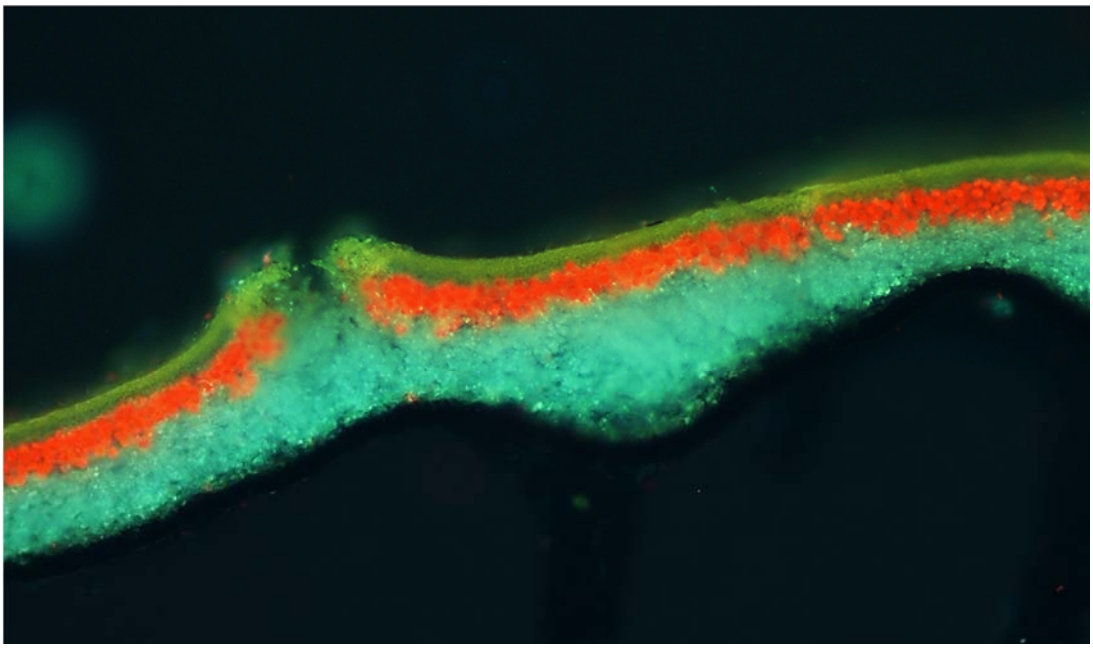

Fig. S1. *Parmelia sulcata* cross section with a pseudocyphellae. The autofluorescence (block UV-2A) reveal a yellow cortical layer of atranorine, a red algal layer in the upper medulla, and a bluish layer of salazinic acid across all the medulla. Lower cortex and rhizines lacks fluorescence.

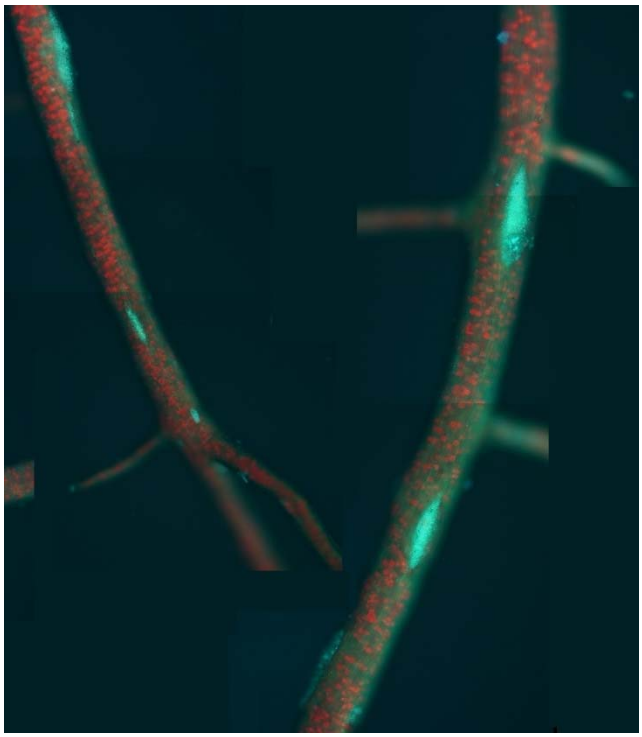

Fig. S2. Autofluorescence (block UV-2A) image of branches of few milimeters from *Bryoria bicolor*, obtained joining partial pictures. Pseudocyphellae, which lacks extrolites detectable by TLC, show a bright bluish fluorescence.

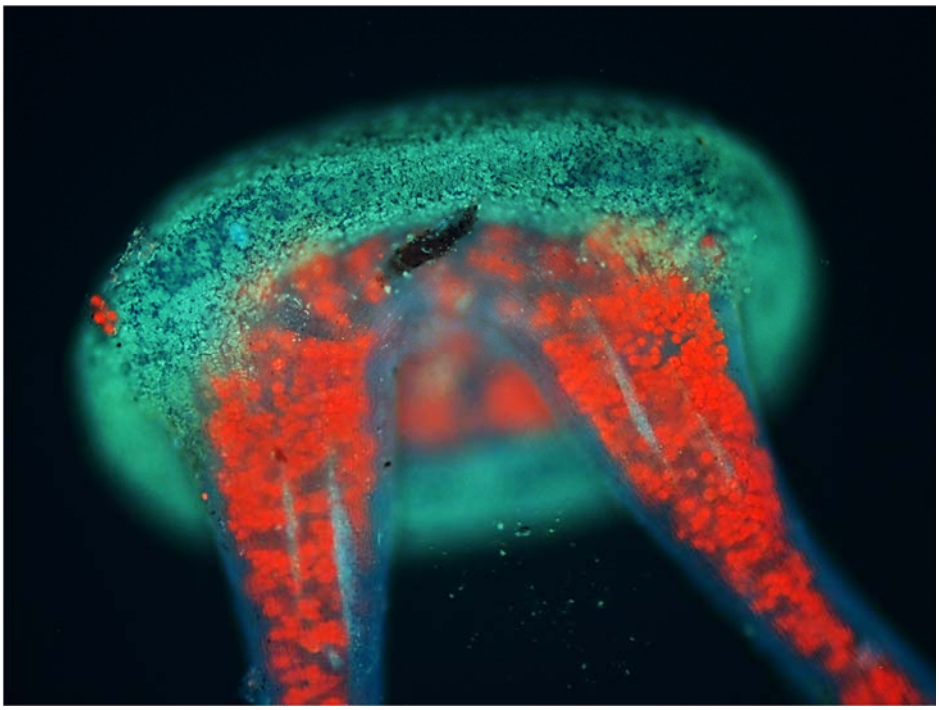

Fig. S3. Autofluorescence (block UV-2A) of a *Bryoria capillaris* apothecia from a lower vision. Apothecial margin (bluish ring) contains an extra-cortical substance not present in the thallus. Algae appear in red.

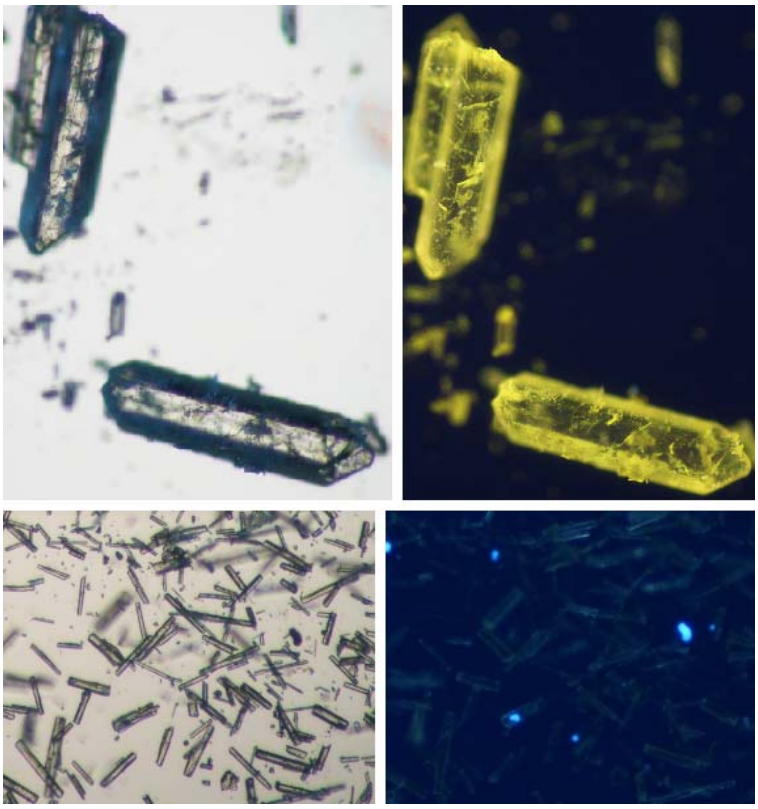

Fig. S4. Pure crystals of atranorine (up) and usnic acid (down) showed without and with fluorescence (block UV-2A). Usnic acid lacks fluorescence under UV light of 450–490 nm.

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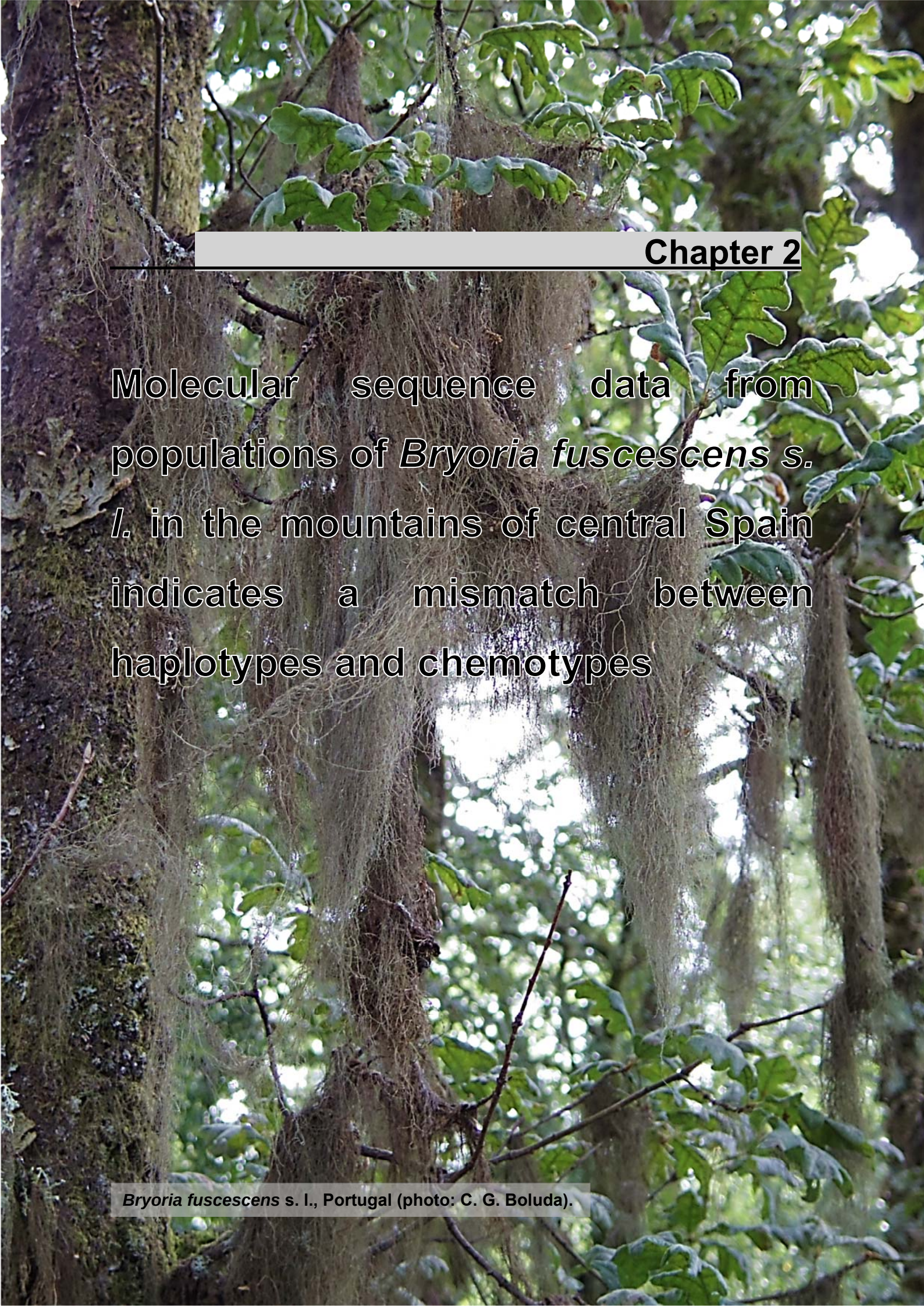
Chapter 2

Molecular sequence data from populations of *Bryoria fuscescens s. l.* in the mountains of central Spain indicates a mismatch between haplotypes and chemotypes



***Bryoria fuscescens* s. l., Portugal (photo: C. G. Boluda).**

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Abstract

In order to confirm and investigate the extent of reported mismatches between chemotypes and molecular sequence data in *Bryoria fuscescens* s. l., we examined 15 morphologically similar thalli from each of three *Pinus* forest sites in the Sistema Central of central Spain. Three thalli were rejected due to infections by *Phacopsis huuskonenii* [1](not previously published from Spain). The remaining 42 thalli represented nine ITS rDNA haplotypes and four chemotypes (by TLC): fumarprotocetraric and protocetraric acids; norstictic and connorstictic acids; psoromic acid; and fumarprotocetraric, protocetraric and psoromic acids. The molecular phylogenetic tree was characterized by extremely short branch lengths, often only with a single mutational difference, and a single haplotype could have different chemical products. In some cases, adjacent specimens represented different chemotypes, and three thalli appeared to be mixed individuals. Consistency of both molecular and chemical data within individual specimens was demonstrated by examining four different parts of each thallus, which showed only a difference in the location of psoromic acid in some. This is the first population-level study of this taxon, and so it is premature to propose taxonomic changes at this time. Further populations in different parts of the geographical range of this widespread complex now need to be analyzed, and more sensitive chemical analyses conducted, in order to understand the basis of the variability and determine the appropriate taxonomic treatment.

Introduction

Bryoria fuscescens s. l., as understood in Europe, is a taxonomically difficult group composed of morphologically similar lichens which have been named as *B. chalybeiformis*, *B. fuscescens*, *B. implexa*, *B. lanestris*, and *B. subcana*. Although chemical and morphological features have differentiated each of these species, specimens with intermediate characters are frequent and prevent confident identifications. Preliminary molecular phylogenetic studies on material morphologically conforming to *Bryoria fuscescens* from the mountains in central Spain and Turkey, conducted in 2007, suggested a mismatch with the chemotypes as revealed by thin-layer chromatography (Hawksworth *et al*. 2011). Independently, Myllys *et al*. (2011a), from studies based on material from a wide geographical range rather than discrete populations, reported a similar mismatch and obtained an unresolved phylogenetic tree for some *Bryoria* sect. *Implexae* species, and subsequently suggested that many of the

[1]As *Raesaenenia huuskonenii* in the next chapters.

recognized species were conspecific (Myllys *et al*. 2011 b). Chemistry has traditionally been emphasized in species separations in *Bryoria*, and was used in major treatments in the 1970s (e.g. Brodo & Hawksworth 1977). The prevailing view, as noted by Krog (1980), was that "morphological plasticity in the genus *Bryoria* makes it necessary to focus on chemical characters in the final delimitation of the species". By the late 1980s, however, it was starting to become evident that some chemotypes should not be separated as different species (Holien 1989). Certain specimens, however, are now being found which contain a range of extrolites[2], such as psoromic, norstictic or fumarprotocetraric acids, which are chemically very similar. Caution in the use of structurally very similar compounds in species separation has previously been expressed (Hawksworth 1976; Lumbsch 1998). Molecular sequence data now afford a method of assessing phylogenetic relationships independently from morphological and chemical characters. As experience with other lichens in *Parmeliaceae* has demonstrated, intensive sampling of populations is necessary to fully understand their variability (e.g. Del-Prado *et al*. 2011). In order to determine whether chemistry was indeed a robust character for species delimitation in *Bryoria fuscescens* s. l., we investigated the relationship between chemotype (the suite of extrolites in a specimen) and genetic kinship as inferred by the ITS rDNA sequences in three populations conforming morphologically to *Bryoria fuscescens* s. str., from the mountains of the Sistema Central in Spain. Furthermore, in order to ascertain if there was variation in the chemical products detected in different parts of the specimens, or if single specimens were of intermixed genotypes or "mechanical hybrids" (Hawksworth 1988), we separated each specimen into four different portions which were examined separately.

Materials and Methods

Three populations conforming morphologically to *Bryoria fuscescens* s. str., collected in the Sistema Central Mountains of Spain, were studied. All specimens sampled had dark coloured thalli, paler basal parts, acute branching angles, pseudocyphellae that were inconspicuous or absent, and fissural as well as tuberculate soralia. In collecting, care was taken to avoid other *Bryoria* species or specimens with different morphological characteristics:

1) *Segovia*: La Granja de San Ildefonso, Sierra de Guadarrama, between Puerto de Cotos and Puerto de Navacerrada, 40°47'34·97"N / 03°59'12·62"W, 1854m, 25 May 2012, *C.*

[2]"Extrolite" refers to all compounds which are secreted from fungal hyphae, and was first used by Frisvad (2005). "Secondary metabolites" is an inappropriate term as these are not metabolized but are often products with ecological roles.

G. Boluda & V. J. Rico (MAF-Lich.18863,18865,18923-18932; GenBank accession numbers KJ652402 to KJ652413).

2) *Madrid*: Navacerrada, Sierra de Guadarrama, La Barranca, 40°46'06·3"N / 03°59'04"W, 1580m, 11 July 2012, *C. G. Boluda & V. J. Rico* (MAF-Lich. 18862, 18933-18946; GenBank accession numbers KJ652414 to KJ652428).
3) *Ávila*: Navarredonda de Gredos, Sierra de Gredos, Pinar de Navarredonda, near the Parador Nacional de Gredos, 40°21'10"N / 05°06'45"W, 1550m, 1 July 2012, *V. J. Rico* (MAF-Lich. 18947 to 18961; GenBank accession numbers KJ652429 to KJ652443).

All three sites were of uneven-aged *Pinus sylvestris* forests over granite, and the specimens were restricted to mature *P. sylvestris* trunks. The lichen community belonged to the *Pseudevernion furfuraceae* (James *et al*. 1977), and was dominated in these sites by *Hypogymnia farinacea*, *Parmelia serrana*, *P. sulcata*, *Platismatia glauca*, *Pseudevernia furfuracea*, and less abundantly *Tuckermannopsis chlorophylla*.

Fifteen discrete specimens were collected in each site. Three of those from the Madrid locality, however, were subsequently rejected due to the presence of the lichenicolous fungus *Phacopsis huuskonenii*, a species not previously published as occurring in Spain. For each of the remaining 42 samples, four thallus regions were cut and examined separately: 1) the base (the oldest part, usually in contact with the bark); 2) the median zone (middle of the branches, but with soralia removed); 3) the tips (the last 5mm of the branches); and 4) the soralia. In total, 168 subsamples (42 × 4) were analyzed, using the same material for TLC and DNA extraction. For the phylogenetic tree reconstruction, *Bryoria glabra* (Finland, GenBank accession number HQ402725.1) was used as outgroup (Myllys *et al*. 2011a).

Extrolite chemistry

Spot tests were made using C, K, KC, and Pd, and TLC was performed using standard methods (Orange *et al*. 2010). For the TLC, concentrated lichen extractions in acetone were spotted onto silica gel 60 F254 aluminium sheets (Merck, Darmstadt) and run with the solvents A, B, C and G. Spots were visualized under UV and after a sulphuric acid spray.

Molecular and bioinformatics techniques

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Barcelona) with a slight modification to the manufacturer's instructions (Crespo *et al*. 2001). The fungal ITS rDNA region was amplified using the primers ITS1FKYO2 (TAGAGGAAG TAA AAG TCG TAA) and ITS4KYO2 (RBT TTC TTT TCC TCC GCT) (Toju *et al*. 2012). For amplification, we used a reaction mixture of 25 µl, containing 18 µl of sterile water, 2.5 µl of 10× buffer with 2 mM $MgCl_2$, 0.5 µl dNTPs (10 mM of each base),1.25 µl of each primer at 10 µM, 0.625 µl of DNA

polymerase (1U μl^{-1}), and 5 µl of diluted 1/10 DNA template. For any failed samples the PCR was repeated using PuReTaq Ready-To-Go PCR Beads (2.5 U of PuReTaq DNA Polymerase, 200 µM of each dNTP, BSA, buffer reaction and stabilizers: 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl2; GE Healthcare, Little Chalfont, UK), adding to the lyophilized bead 20 µl of sterile water, 1 µl of each primer at 10 µM and 3 µl of diluted 1/10 DNA template.

The amplifications were run in an automatic thermocycler (XP Cycler, Bioer, Hangzhou) using the following parameters: initial denaturation 5 min at 95 °C, then 35 cycles of 1 min at 95 °C,1 min at 56 °C,1.5 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products were cleaned using illustra™ ExoProStar (GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. Sequencing was performed by the Unidad de Genómica (Parque Científico de Madrid) and Stabvida (Lisbon, Portugal).

DNA sequences obtained were manually adjusted using SeqMan version 7.0 (DNAstar, Madison) and MEGA5 (Tamura *et al*. 2011). For genetic analyses, only one sequence per specimen instead of four was used, selecting the sequences belonging to the basal portion as those would be of the haplotype when the thallus started to grow. The alignment was performed using MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/; Katoh & Standley 2013), using G-INS-I alignment algorithm, a scoring matrix of 1PAM/k = 2, and offset value of 0.1. Gblocks version 0.91b (Barcelona; http://molevol.cmima.csic.es/castresana/Gblocks_server.html) was used to delete non-conserved GAPs, allowing smaller final blocks, gap positions within the final blocks, and less strict flanking positions, which resulted in the elimination of a single gap in the outgroup. The alignment was analyzed using maximum likelihood (ML) and Bayesian (B/MCMC) approaches. For the maximum likelihood (ML) tree reconstruction, we used the program RAxML v.7.2.8 (Stamatakis 2006). The GTRGAMMA model was applied, which includes a parameter (Γ) for rate heterogeneity among sites, and we chose not to include a parameter for estimating the proportion of non-variable sites (Stamatakis 2006; Stamatakis *et al*. 2008). Analysis was performed using RAxML v.7.2.8, as implemented on the CIPRES Science Gateway (http:// qball2.sdsc.edu:7070/portal2/home.action; Miller *et al*. 2010) with the GTRGAMMA model as described above. Support values were assessed using the 'rapid bootstrapping' option with 1000 replicates. For the Bayesian reconstruction, MrBayes v.3.2.1 (Ronquist & Huelsenbeck 2003) was used. The analysis was performed assuming the general time reversible model (Rodríguez *et al*. 1990), assuming a discrete gamma distribution with six rate categories (GTR+G). The nucleotide-substitution model and parameters were selected using the Akaike Information Criterion as implemented in jModelTest (Posada 2008). A run with four million generations, starting with a random tree and employing eight simultaneous chains, was executed. Every 400th tree was saved to a file. We plotted the log-likelihood scores of sample

points against generations using TRACER v.1.5 (Rambaut & Drummond 2007) and determined that stationarity had been achieved when the log-likelihood values of the sample points reached an equilibrium value (Huelsenbeck & Ronquist 2001), discarding the trees obtained before stationarity was reached. Posterior probabilities (PPs) were obtained from the 50 % majority-rule consensus of sampled trees after excluding the initial 25 % as burn-in. The phylogenetic tree was drawn using FigTree v.1.4 (Rambaut 2009).

For the haplotype network reconstruction, TCS v1.2.1 was employed, using gaps as missing data and 95 % as the connection limit. We used DnaSP v.4.50 (Librado & Rozas 2009) to calculate estimates of genetic diversity. PAUP 4.0 was used to calculate the haplotype diversity, number of polymorphic sites, nucleotide diversity, Tajima's D value, Fu's F statistic, and the raggedness index. For genetic comparison among predefined groups, the AMOVA test was implemented in Arlequin v.3.5 (Excoffier *et al*. 2005), comparing differences among chemotypes and among populations. In order to test if there was a definite correlation between chemotypes and haplotypes, we performed a Fisher's exact test implemented using the Rpackage (R Development Core Team 2012).

Results

Chemical investigations

Four extrolite profiles were found: 1) norstictic and connorstictic acids; 2) fumarprotocetraric, protocetraric, and psoromic acids; 3) protocetraric and fumarprotocetraric acids only; and 4) psoromic acid only (Table 1). We did not detect atranorine in this study; this compound is known to occur sporadically in various *Bryoria* species (e.g. Myllys *et al*. 2011b) but is generally at low concentrations and not found in routine TLC. Although the three populations were in the same macro-environment, there were marked differences in the percentage abundance of each chemotype. In some cases, two specimens collected close together, and apparently in the same micro-environment, had different extrolite profiles.

TLC did not reveal differences in the presence/absence of extrolites in the four thallus regions, nor between the soralia and other parts of the thallus, except in two specimens. One had protocetraric, fumarprotocetraric, and psoromic acids in all parts of the thallus, except that the base lacked psoromic acid, while the other contained protocetraric and fumarprotocetraric acids except for the soralia which additionally contained psoromic acid. Although TLC indicates a homogeneous extrolite distribution along the thallus (with the exception of these two specimens), this may not be conclusive, as autofluorescence studies indicate that there can

be chemical heterogeneity within thallus portions (Boluda *et al*. 2014). For example, norstictic acid is commonly only present in soralia and inconspicuous pseudocyphellae, while fumarprotocetraric acid can be restricted to soralia. TLC of acetone extracts from thallus portions cannot detect such small-scale heterogenic distributions. The results in the current study therefore have to be interpreted as indicating that, while there is generally no variation in extrolite composition from the base to the tips, they cannot exclude the possibility of heterogeneous small scale distribution within thallus portions.

Table 1. Chemotype frequencies in the 42 specimens of *Bryoria fuscescens* examined from 3 separate populations collected in 3 localities.

	Chemotype frequences			
Locality	**N**	**F**	**P**	**FP**
Segovia	7	2	6	0
Madrid	4	8	0	0
Ávila	2	1	10	2

N = norstictic and connorstictic acids, F = fumarprotocetraric and protocetraric acids, P = psoromic acid, FP = fumarprotocetraric, protocetraric and psoromic acids.

Molecular investigations

The amplified PCR products obtained were around 800 bp. Usually, this PCR product is about 600 bp; the difference in size found in our samples was due to the presence of insertions of about 200 bp identified as group I introns (Gutiérrez *et al*. 2007) at the 3' end of the SSU rDNA. We excluded group I introns as well as the SSU and LSU neighbouring regions of the ITS from the analysis. The ITS sequences of the four thallus portions of each of the 42 specimens (42 × 4) revealed three cases of intra-thalline diversity (7.14 % of the samples). These specimens were composed of two different genotypes, which were also present in other specimens from the same populations. In one specimen, the genotypes alternated among the four thallus portions, but in the other two the median zone contained a different genotype. In these three specimens, the extrolites were the same in all four segments tested. This result

suggests that what seems to be a discrete and independent thallus can be composed of two or more intermixed genotypes.

The sequences used for tree and haplotype network reconstruction (one per specimen) contained a haplotypic diversity (Hd) of 0.850, seven polymorphic sites (S), a nucleotide diversity (π) of 0.00331, a Tajima's D value of −0.4847 ($P > 0.10$, not significant), a Fu's F statistic of −2.519 ($P > 0.10$, not significant), and a raggedness index (R) of 0.0944 ($P > 0.10$, not significant) with a unimodal mismatch distribution. Each of the 42 specimens was found to belong to one of nine haplotypes. The tree reconstruction (Fig. 1) was characterized by exceptionally short branch lengths (note that the scale in Fig. 1 = 0.003 substitutions per site) compared with those seen within other species of the genus (cf. Myllys *et al*. 2011a). This represents a particularly low level of genetic diversity in the ITS sequences, many specimens having identical sequences. The same haplotypes occurred in the different populations, as evident from the haplotype network obtained (Fig. 2); all haplotypes were connected by a single mutation. Three of the nine haplotypes were represented by single specimens. Haplotypes represented by more than one specimen (except haplotype 2 with two specimens), also included more than one chemotype. Three of the predominant haplotypes (numbers 5, 9 and 6, with nine, nine and five specimens, respectively) contained specimens showing in total the full range of extrolites found in this study within each haplotype (norstictic, psoromic, and fumarprotocetraric acids). AMOVA results showed that 24.7 % of the genetic variation was among the four chemotypes, while the variation among the collected populations was 7.8 %.

The Fisher's exact test indicated that the hypotheses of there being no correlation between haplotypes and chemotypes ($P < 0.001$), nor between haplotypes and populations ($P < 0.005$), could not be entirely ruled out from the data available. The reason for this was that some haplotypes were represented by only one or two specimens in only one chemotype or population. In the case of the haplotypes in our study represented by greater numbers of specimens, the test showed that haplotypes and chemotypes were not correlated. This suggests that there is no correlation between extrolite chemistry and genetic kinship, but more samples are needed to be confident that this is the case for all haplotypes and chemotypes represented in our samples.

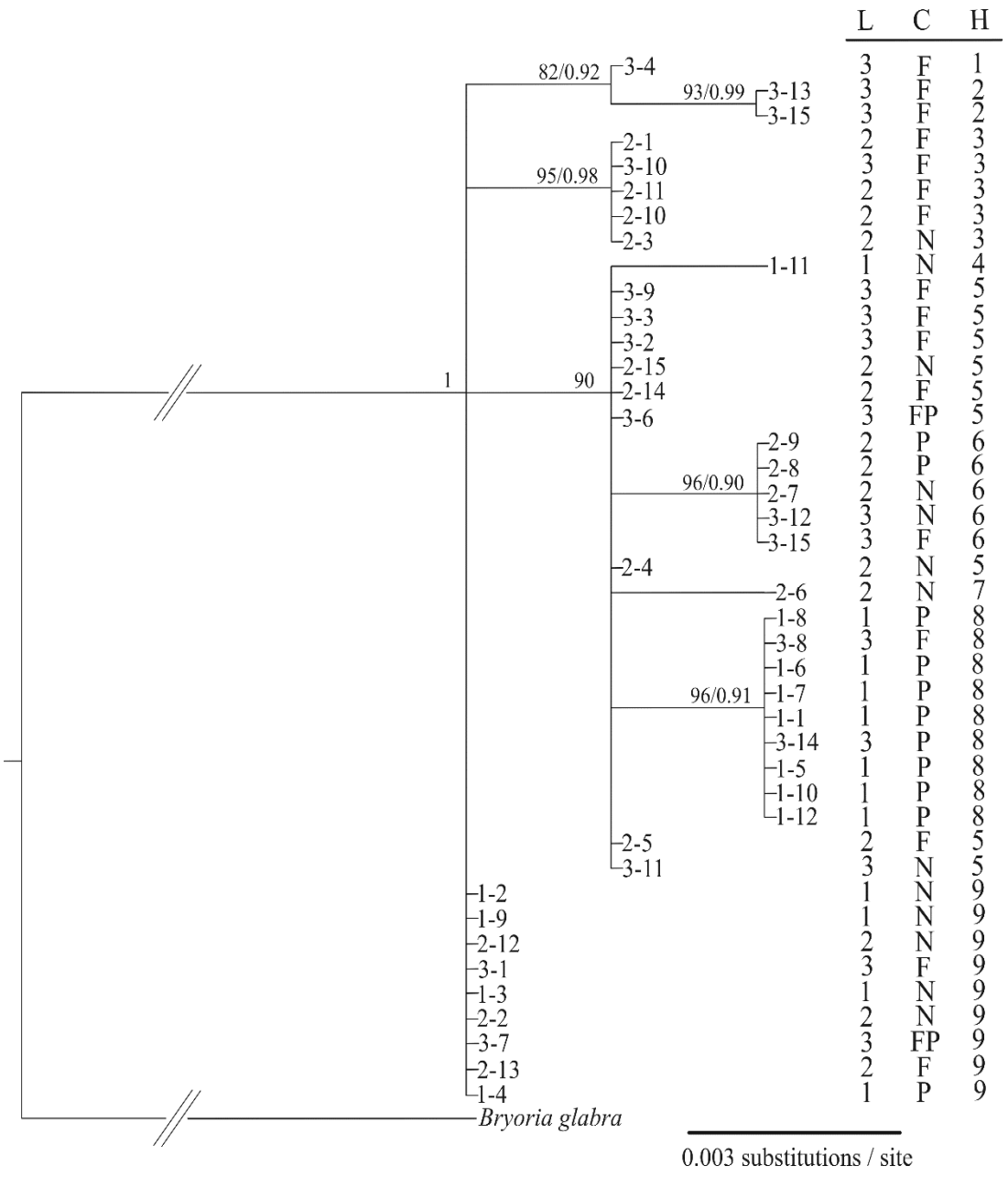

Fig. 1. Phylogram obtained from maximum likelihood analysis of ITS rDNA sequences from 3 populations of *Bryoria fuscescens* obtained from the Sistema Central Mountains of Spain. ML bootstrap values ≥ 75 and posterior probabilities ≥ 0.90 for the Bayesian analyses are indicated above the branches. The tree tip numbers indicate the locality and specimen number respectively. L = locality (1 = Segovia, 2 = Madrid, and 3 = Ávila); C = chemotype (F = fumarprotocetraric and protocetraric acids, N = norstictic and connorstictic acids, P = psoromic acid, FP = fumarprotocetraric, protocetraric and psoromic acids); H = haplotype.

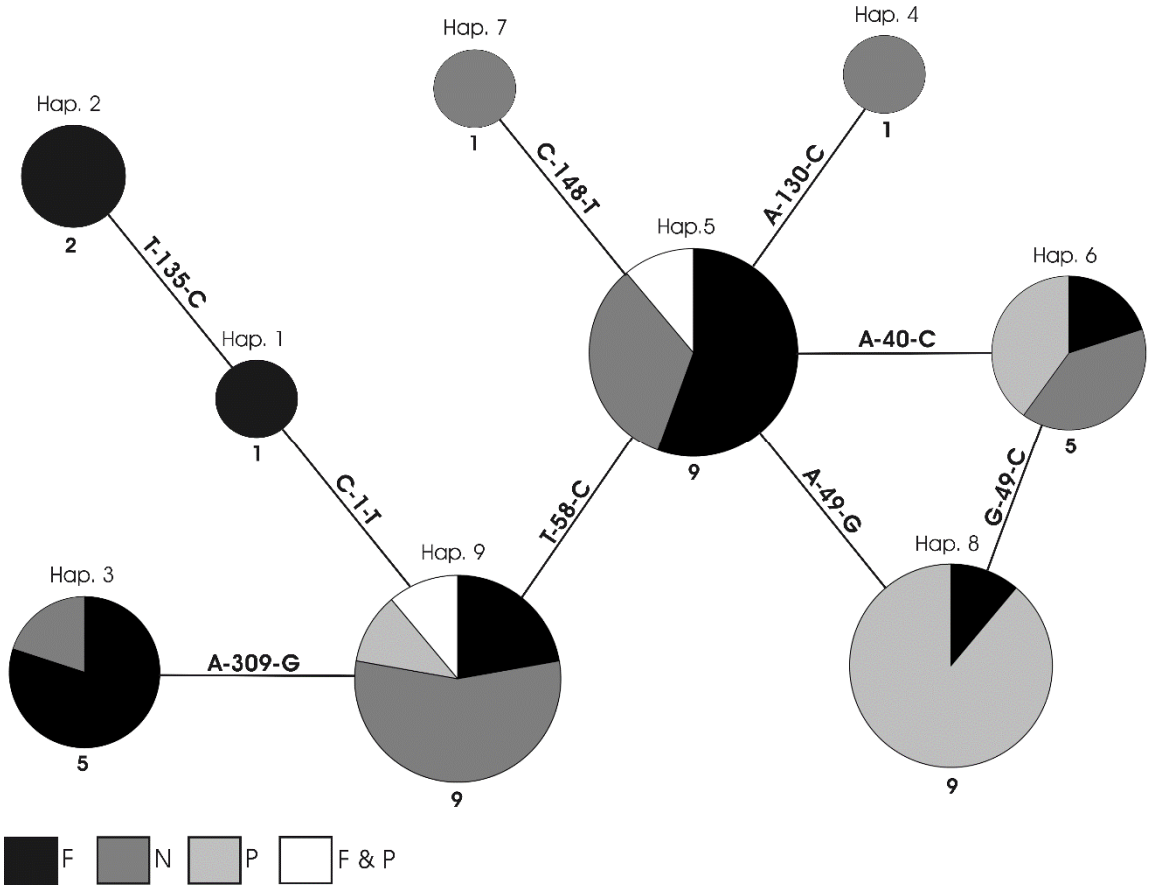

Fig. 2. 95% probability haplotype network based on ITS rDNA sequences of the 42 specimens from 3 populations of *Bryoria fuscescens* examined. The circle size is proportional to the number of specimens sharing a haplotype. Numbers below the circles indicate the number of specimens. Text on the connecting lines indicates the nucleotide substitution. Hap. = haplotype, F = fumarprotocetraric and protocetraric acids, N = norstictic and connorstictic acids, P = psoromic acid.

Discussion

Bryoria fuscescens s. l. is taxonomically difficult to resolve and the assignment of specimens to currently recognized species can be frustrating. Our results, the first to be based on intensive molecular and chemical analyses of discrete morphologically more or-less uniform populations conforming to *B. fuscescens* s. str. but including some deviating chemically, suggest that extrolite production, which has been emphasized in species circumscription in these lichens since the 1970s, is not correlated with particular haplotypes (phylogenetic lineages) as revealed by ITS. We also show that populations lacking apothecia and so expected to reproduce clonally, and which also appear morphologically homogeneous, can

comprise a mixture of haplotypes and can vary in the TLC-detectable extrolites. We found that specimens morphologically assigned to *B. fuscescens* may not contain fumarprotocetraric acid only, as historically assumed, but are much more variable in their chemistry. As pointed out previously (Boluda *et al*. 2014), however, a fuller picture of the extrolite patterns in the complex will require the use of high performance liquid chromatography (HPLC) to detect compounds present at lower concentrations than can be visualized by TLC from extracts of single small thallus portions, and further fluorescence microscopy to explore the localization of compounds in thalli more precisely. In addition, negative but non-significant Tajima's D value and Fu's F statistics may indicate population stability (i.e. no evidence of demographic expansion or contraction) of *B. fuscescens* in the regions studied. The unimodal curve of mismatch frequency, however, suggests population expansion or spatial range expansion, but with a non significant raggedness index. These contradictory results may well be attributable to bias arising from the limited sample size (Ramos-Onsins & Rozas 2002), and so more comprehensive studies would be required to test that hypothesis.

We conclude that extrolite composition appears to be of limited value for the possible separation of species within material conforming morphologically, but not always chemically, to *Bryoria fuscescens*. Furthermore, as the three populations we investigated were growing in similar ecological situations and on the same species of tree, there were no evident ecological factors to which the differences observed could be attributed, nor were there associations with particular genetic lineages as revealed by the ITS rDNA region.

Similar population studies across the range of the species complex are required to determine the extent to which those of the Spanish Sistema Central are representative of the situation throughout its geographical range. Furthermore, *B. fuscescens* s. l. is much more abundant in northern Europe, where apothecia can sometimes be found, albeit at a low frequency, while in southern Europe specimens are almost exclusively asexual and occur as isolated populations. It would therefore be of interest to ascertain if a greater range of genetic diversity occurs in more northern regions.

Without more detailed population studies over a larger geographical area, we consider it unwise to conclude that material currently named as *Bryoria capillaris*, *B. chalybeiformis*, *B. fuscescens*, *B. implexa*, *B. lanestris*, or *B. subcana* should be treated as conspecific on the basis of DNA data alone, as suggested by Myllys *et al*. (2011b). In order to elucidate the situation in *Bryoria fuscescens* s. l., and lead to a more robust taxonomy for these lichens, sequences from more DNA regions and microsatellite population studies are required to determine if there are other correlations between chemistry, morphology, geography, and genetic data.

Acknowledgements

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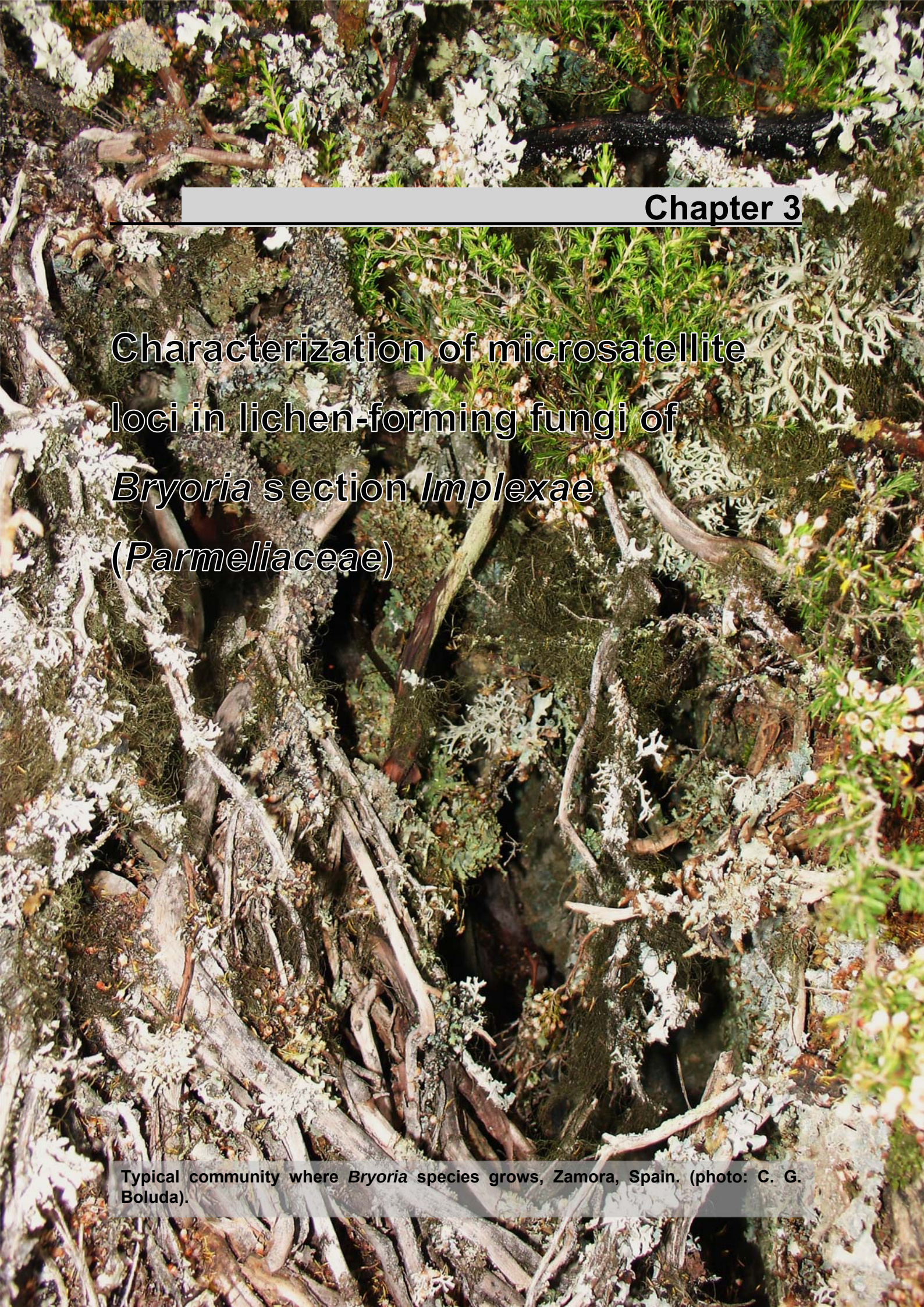

Chapter 3

Characterization of microsatellite loci in lichen-forming fungi of *Bryoria* section *Implexae* (*Parmeliaceae*)

Typical community where *Bryoria* species grows, Zamora, Spain. (photo: C. G. Boluda).

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Abstract

The locally rare, haploid, lichen-forming fungi *Bryoria capillaris*, *B. fuscescens*, and *B. implexa* are associated with boreal forests and belong to *Bryoria* sect. *Implexae*. Recent phylogenetic studies consider them to be conspecific. Microsatellite loci were developed to study population structure in *Bryoria* sect. *Implexae* and its response to ecosystem disturbances. We developed 18 polymorphic microsatellite markers using 454 pyrosequencing data assessed in 82 individuals. The number of alleles per locus ranged from two to 13 with an average of 4.6. Nei's unbiased gene diversity, averaged over loci, ranged from 0.38 to 0.52. The markers amplified with all three species, except for markers Bi05, Bi15, and Bi18. The new markers will allow the study of population subdivision, levels of gene introgression, and levels of clonal spread of *Bryoria* sect. *Implexae*. They will also facilitate an understanding of the effects of forest disturbance on genetic diversity of these lichen species.

Introduction

The members of *Bryoria* sect. *Implexae* are pendent, copiously branched lichens with circumboreal distribution (Brodo & Hawksworth 1977; Myllys *et al*. 2011a). They are an important component of the boreal forests (Glavich *et al*. 2005), and their frequency depends on forest fragmentation (Hilmo & Holien 2002). These lichen-forming fungi are haploid and disperse with vegetative propagules; sexual reproduction with ascospores is uncommon (Brodo & Hawksworth 1977). *Bryoria* sect. *Implexae* includes seven morphologically and chemically recognized species in Europe (Myllys *et al*. 2011a), which have different frequency across longitudinal and altitudinal gradients (Hawksworth 1972; Myllys *et al*. 2011a). Molecular data confirm the monophyly of the section, although the relationships among the currently recognized species remain poorly understood because phylogenetic analyses suggest that several species are conspecific (Myllys *et al*. 2011b). Highly variable microsatellite markers of the fungal partner of lichen symbioses (Widmer *et al*. 2010; Devkota *et al*. 2014) will be used to study the genetic diversity and differentiation in *Bryoria* sect. *Implexae*, to determine the gene flow across and within the currently recognized species, and to assess the impact of land use and habitat fragmentation on population structure of these locally rare and threatened, boreal forest–associated lichens.

Methods and results

Eighty-two specimens representing the three morphologically and chemically characterized species, *Bryoria capillaris* (Ach.) Brodo & D. Hawksw., *B. fuscescens* (Gyeln.) Brodo & D. Hawksw., and *B. implexa* (Hoffm.) Brodo & D. Hawksw., were collected in three regions (Spain, Switzerland, and Finland; Appendix 1). All specimens are deposited in the Lichens Herbarium of the Universidad Complutense de Madrid (MAF-Lich.), and duplicates are stored at the Swiss Federal Research Institute WSL at −20 °C. A subset of 30 specimens was used for total DNA extraction with the MoBio PowerPlant Pro DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA). The pooled DNA was used to create a shotgun multiplex identifier library using the GS FLX Titanium Rapid Library Preparation Kit (Roche Diagnostics, Basel, Switzerland), and Microsynth AG (Balgach, Switzerland) provided the barcode adapters. The library was sequenced on 1/4th of a plate on a Roche 454 Genome Sequencer FLX at Microsynth. We obtained 533,962 reads of an average length of 812 bp (National Center for Biotechnology Information [NCBI] Sequence Read Archive [SRA] accession no. SRR1283191; http://www.ncbi.nlm.nih.gov/sra). The unassembled sequences were screened for di-, tri-, tetra-, and pentanucleotide microsatellites using MSATCOMMANDER 1.0.2 alpha (Rozen & Skaletsky 1999; Faircloth 2008), ensuring a minimum repeat length of 8 bp for dinucleotides and 6 bp for all others.

MSATCOMMANDER recovered 6329 primer pairs that fulfilled the default primer parameters among all reads. Of those, 5932 pairs were discarded from further studies because they contained unfavorable secondary structure, primer-dimer formation, monorepeats in the flanking region, or because they were duplicates, which we detected after alignment using CLC Main Workbench 6 (CLC bio, Aarhus, Denmark). Putative sequences of algae, plants, animals, or microorganisms, which are often present in epiphytic samples, were identified and removed using the ntBLAST search on http://www.ncbi.nlm.gov. This inspection resulted in 58 primer pairs used for further analysis, i.e., to test for amplification with the symbiotic partner of these lichen-forming fungi. We used DNA from five axenic cultures of *Trebouxia* spp., which are hypothesized to be the photobionts of *Bryoria* sect. *Implexae* (Lindgren *et al*. 2014): *Trebouxia angustilobata* Beck (SAG2204), *T. asymmetrica* Friedl & Gärtner (SAG48.88), *T. arboricola*

Tabla 1. Overview of the microsatellite loci developed for the group of lichen-forming fungi *Bryoria* sect. *Implexae*.

Locus	Primer sequences (5'-3')	Repeat motif	Multiplex[a]	T_a (ºC)	Fluorescent dye	Primer conc. (µM)	Allele size range (bp)	GenBank accession No.
Bi01	F: GGACGACGACATACCACTC	$(AACAGC)_6$	1	56	FAM	0.32	94-129	KJ739845
	R: GAGTTCGGGTTTAGGTCGTC							
Bi02	F: GCGTGAATGTGTCCGAATCG	$(AG)_{12}$	1	56	FAM	0.80	369-372	KJ739846
	R: GAATGGGCGCTCACTGTCTT							
Bi03	F: GTGAACTCGCTCGTATCGTC	$(AG)_{12}$	1	56	FAM	0.80	279-281	KJ739847
	R: CCTAGGGATGACACGCAGAA							
Bi04	F: CAGTGCGGCAAACAGTTAGT	$(TG)_{10}$	1	56	PET	0.80	320-325	KJ739848
	R: GCACAAATCCACCCACTCCT							
Bi05	F: CAAGGAGGTCGACTGTGAGT	$(AAGG)_6$	1	56	NED	0.50	127-143	KJ739849
	R: CAACCGATCCCACGCTCTC							
Bi06	F: GGGAGGGTGGAAGTTGGTTT	$(GTT)_9$	1	56	PET	0.32	114-168	KJ739850
	R: CGACCACTTCCACTTCCATATC							
Bi07	F: GAAATCGGCTTGTTGTCCTCC	$(CCTTT)_6$	2	58	PET	0.80	123-144	KJ739851
	R: GAACTACCGCCCACAAACAA							
Bi08	F: CATGCGGAGTTAAAGGAGGC	$(TC)_8$	2	58	NED	0.32	367-372	KJ739852

	R: CGCACCTATTTACGGCCTTT							
Bi09	F: CGTTCGTTCGTAGGTAGGTA	$(AT)_8$	2	58	PET	1.10	341-343	KJ739853
	R: GCCTACCCACCATCTGAACT							
Bi10	F: CTCGCGTTTCCCTGTTTCTT	$(TC)_8$	2	58	FAM	0.90	434-437	KJ739854
	R: GTATGAGGTCGGAGTGTGCT							
Bi11	F: GCACAAATCCACCCACTCCT	$(AC)_{12}$	2	58	FAM	0.50	314-318	KJ739855
	R: CAGTGCGGCAAACAGTTAGT							
Bi12	F: GCAGAAAGTGAGTTAGCCGG	$(TTG)_{12}$	2	58	FAM	0.32	100-124	KJ739856
	R: CTCAGCCTCAACCACAACGA							
Bi13	F: TCTTTCCTCTCCTGTCCACC	$(TTC)_{11}$	3	60	FAM	0.90	93-134	KJ739857
	R: CCTTACAGACCGGAGAAGCC							
Bi14	F: CTAACCACGACAAGCTGACC	$(TC)_7$	3	60	FAM	0.60	316-365	KJ739858
	R: GTACCGACGCAACTTACCTA							
Bi15	F: GTAGCAGGACATACGGAGGT	$(TC)_9$	3	60	PET	3.00	379-381	KJ739859
	R: CGTCCTAGCATCTCGGTTCT							
Bi16	F: CCAGGTCCTTCACTACAGCT	$(AG)_8$	3	60	FAM	1.50	405-437	KJ739860
	R: CGGTACAAGTCCAGTTGCAG							
Bi18	F: GCAGCTATCAGGAGTCACGT	$(TC)_7$	3	60	VIC	0.60	387-396	KJ739861

	R: GCAGCTATCAGGAGTCACGT							
Bi19	F: CCACCTCGAAGAGTACTGCT	$(TC)_{10}$	3	60	PET	0.80	346-352	KJ739862
	R: CTGAGCTATGTCCTCGCACA							

Note : T_a = annealing temperature.
[a]Multiplex indicates loci that were mixed in the same capillary electrophoresis run.

Table 2. Results of microsatellite screening in 82 individuals of lichen-forming fungi of *Bryoria* sect. *Implexae* between species of *Bryoria* sect. *Implexae*, and between compared regions.

	Total			***Bryoria capillaris*** **(n = 36)**		***B. fuscescens*** **(n = 37)**		***B. implexa*** **(n = 37)**		**Spain (n = 31)**		**Switzerland (n = 35)**		**Finland (n = 16)**	
Locus	**n**	**A**	**H_e**	**A**	**H_e**	**A**	**H_e**	**A**	**H_e**	**A**	**H_e**	**A**	**H_e**	**A**	**H_e**
Bi01	82	7	0.82	6	0.71	6	0.79	4	0.58	5	0.73	6	0.71	4	0.44
Bi02	67	4	0.74	3	0.64	4	0.68	2	0.43	3	0.68	4	0.69	3	0.59
Bi03	82	2	0.24	2	0.32	2	0.15	2	0.22	2	0.12	2	0.36	2	0.13
Bi04	82	3	0.36	2	0.45	2	0.28	2	0.39	2	0.12	3	0.54	2	0.33
Bi05	79	4	0.61	3	0.57	3	0.47	2	0.22	3	0.52	4	0.66	2	0.13
Bi06	82	10	0.83	5	0.88	5	0.64	3	0.64	3	0.53	8	0.85	4	0.64
Bi07	82	3	0.49	2	0.37	2	0.11	1	0.00	2	0.28	3	0.46	2	0.13

Bi08	82	4	0.54	3	0.52	3	0.49	3	0.56	2	0.49	3	0.54	3	0.57
Bi09	60	2	0.50	2	0.25	2	0.28	1	0.00	2	0.40	2	0.31	2	0.33
Bi10	82	2	0.44	2	0.44	2	0.05	1	0.00	2	0.23	2	0.49	2	0.13
Bi11	82	3	0.36	2	0.45	2	0.28	2	0.39	2	0.12	3	0.54	2	0.33
Bi12	82	7	0.67	5	0.39	6	0.49	4	0.81	3	0.34	6	0.48	5	0.82
Bi13	82	13	0.84	8	0.80	8	0.68	6	0.92	6	0.67	9	0.83	7	0.88
Bi14	82	3	0.47	2	0.40	2	0.05	1	0.00	2	0.23	3	0.48	2	0.13
Bi15	52	2	0.04	2	0.00	2	0.05	1	0.00	1	0.00	2	0.13	1	0.00
Bi16	82	6	0.76	5	0.57	5	0.61	3	0.72	3	0.61	6	0.67	4	0.69
Bi18	81	4	0.56	3	0.35	3	0.62	3	0.68	3	0.59	4	0.27	3	0.68
Bi19	82	3	0.65	3	0.11	3	0.53	3	0.72	3	0.60	3	0.43	3	0.69
Mean		4.58	0.53	6	0.71	6	0.79	4	0.58	2.63	0.38	4.11	0.52	2.84	0.40

Note : A = Number of alleles; H_e = Nei's unbiased gene diversity; n = total number of samples analyzed.

Puymaly (SAG219-1a), *T. jamesii* (Hildreth & Ahmadjian) Gärtner (SAG2103), and *T. simplex* Tschermak-Woess (SAG101.80). Forward primers were labeled with an M13 tag (5′-TGTAAAACGACGGCCAGT-3 ′) for PCR amplification (Schuelke 2000). All PCR runs were performed on Veriti Thermal Cyclers (Life Technologies, Carlsbad, California, USA). The PCR reactions were evaluated in a temperature gradient with one-degree steps from 56–61 °C, performed with the JumpStart REDTaq ReadyMix (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's protocol, with the following conditions: denaturation for 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 56–61 °C, and 45 s at 72 °C; then for the M13-tag binding additional eight cycles of 30 s at 94 °C, 45 s at 53 °C, and 45 s at 72 °C, with a final extension of 30 min at 72 °C. In total, 14 primer pairs produced positive PCR reactions with at least one of the five *Trebouxia* species, and were excluded from further analyses because they were considered alga-specific.

The amplification of the fungal component of *Bryoria* sect. *Implexae* was tested with the 44 remaining loci under the same conditions as mentioned above. There were 14 loci that produced specific single products at an annealing temperature of 56 °C, 12 at 57 °C, six at 58 °C, six at 60 °C, and six at 61 °C. Polymorphism of the 44 microsatellite loci was initially tested on a subset of 12 individuals (four individuals from each of three countries: Spain, Switzerland, and Finland), resulting in 18 polymorphic loci with satisfactory amplification. All PCR products obtained were multiplexed (Table 1). PCR reactions were performed in a total volume of 10 µL containing 1 µL of ~5 ng genomic DNA, 1 µL each of forward and reverse primers of varying concentration (Table 1), and 5 µL of Type-it Multiplex PCR Master Mix (QIAGEN, Hilden, Germany). The PCR protocol used fluorescent forward primers and the reaction was adjusted to: 5 min at 95 °C; followed by 30 cycles of 30 s at 95 °C , 90 s at 56, 58, or 60 °C (Table 1), and 30 s at 72 °C; with a final extension of 60 min at 60 °C. PCR products were run on a 3130xl DNA Analyzer with GeneScan 500 LIZ as the size standard for fragment analysis (both by Life Technologies).

The 18 polymorphic microsatellite markers were tested for locus variability and marker consistency on three populations (Table 2). Alleles were sized using GeneMapper 5.0 (Life Technologies). The linkage disequilibrium (LD) between microsatellite loci and their variability were measured by counting the number of alleles and calculating Nei's unbiased gene diversity using Arlequin 3.11 (Excoffier *et al*. 2005). Dinucleotide microsatellites (n = 13) were the most common microsatellite motifs among the 18 loci (Table 1). The microsatellite loci revealed significant LD based on 999 permutations ($P < 0.001$). They show two to 13 alleles per locus with a mean of 4.6, and average gene diversities varied from 0.38 to 0.52 over three populations (Table 2).

Cross-species amplifications within three congeneric species were analyzed with the chi-square test; *Bryoria capillaris* was shown to not amplify consistently, while *B. fuscescens* and *B. implexa* amplified more regularly (Appendix 2). Most markers amplified with all three species. However, the microsatellite marker Bi15 only amplified with *Bryoria fuscescens*, Bi05 with *B. fuscescens* and *B. implexa*, and Bi18 with *B. capillaris* and *B. fuscescens.*

Conclusions

The fungus-specific markers developed here will facilitate studies on genetic diversity and differentiation in *Bryoria* sect. *Implexae* throughout its geographic distribution, and on effects of forest management on genetic diversity of populations in this species group. Furthermore, putative phylogenetic signal within the flanking regions of the microsatellite sequences might help to delimit closely related species and to assess the taxonomic value of the morphological and chemical characters of these regionally rare and threatened lichens.

Supplementary material

Appendix 1. Voucher information of samples of *Bryoria* sect. *Implexae* used in this study.

***Bryoria* species**	**Voucher No.[a]**	**Collection locality and date**	**Geographic coordinates**	**n**
Bryoria capillaris	18964–18967	Spain, Prov. Segovia, 1854 m a.s.l., *Pinus sylvestris* forest, 6 Nov. 2012.	40°47′35.0″N 03°59′12.6″W	4
B. capillaris	18968–18993	Switzerland, Canton of Berne, 1511 m a.s.l., *Picea abies* forest, 25 Nov. 2012.	46°35′28.3″N 07°20′26.9″E	26
B. capillaris	18997–18999	Finland, Prov. Etelä-Häme, Liesjärvi, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012.	60°40′17.0″N 23°51′10.4″	3
B. capillaris	18994–18996	Finland, Prov. Etelä-Häme, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012.	60°42′04.3″N 23°54′41.9″E	3

B. fuscescens	19001–19014	Spain, Prov. Madrid, 1490 m a.s.l., *Pinus sylvestris* forest, 6 Nov. 2012.	40°46′05.4″N 03°59′35.9″W	14
B. fuscescens	19015–19027	Spain, Prov. Segovia, 1854 m a.s.l., *Pinus sylvestris* forest, 6 Nov. 2012.	40°47′35.0″N 03°59′12.6″W	13
B. fuscescens	19028–19034, 19036	Switzerland, Canton of Berne, 1511 m a.s.l., *Picea abies* forest, 25 Nov. 2012.	46°35′28.3″N 07°20′26.9″E	8
B. fuscescens	19000, 19035	Finland, Prov. Etelä-Häme, Liesjärvi, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012.	60°40′17.0″N 23°51′10.4″E	2
B. implexa	19037	Switzerland, Canton of Berne, 1511 m a.s.l., *Picea abies* forest, 25 Nov. 2012.	46°35′28.3″N 07°20′26.9″E	1
B. implexa	19038–19042	Finland, Prov. Etelä-Häme, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012.	60°42′04.3″N 23°54′41.9″E	3
B. implexa	19043–19045	Finland, Prov. Etelä-Häme, Liesjärvi, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012.	60°40′17.0″N 23°51′10.4E	5

[a]Vouchers deposited at Lichens Herbarium of the Universidad Complutense de Madrid (MAF-Lich.), n = number of individuals.

Appendix 2. Percentage of successful amplification between species of *Bryoria* sect. *Implexae*, and between compared region.

Group	*Bryoria capillaris*	*B. fuscescens*	*B. implexa*	Spain	Switzerland	Finland	Total
n	36	37	9	31	35	16	82
p	0.008	0.81	0.99	0.97	0.86	0.39	-
Bi01	100	100	100	100	100	100	100
Bi02	94	68	89	65	97	81	81-84
Bi03	100	100	100	100	100	100	100
Bi04	100	100	100	100	100	100	100
Bi05	92	100	100	100	91	100	97
Bi06	100	100	100	100	100	100	100
Bi07	100	100	100	100	100	100	100
Bi08	100	100	100	100	100	100	100
Bi09	97	51	67	52	94	69	72
Bi10	100	100	100	100	100	100	100
Bi11	100	100	100	100	100	100	100
Bi12	100	100	100	100	100	100	100
Bi13	100	100	100	100	100	100	100
Bi14	100	100	100	100	100	100	100
Bi15	22	100	78	90	43	56	63-67
Bi16	100	100	100	100	100	100	100
Bi18	100	100	89	100	97	100	96-99
Bi19	100	100	100	100	100	100	100

Note: n = total number of samples analyzed; p = probability (according to chi-square test) that each group will equally amplify with all markers.

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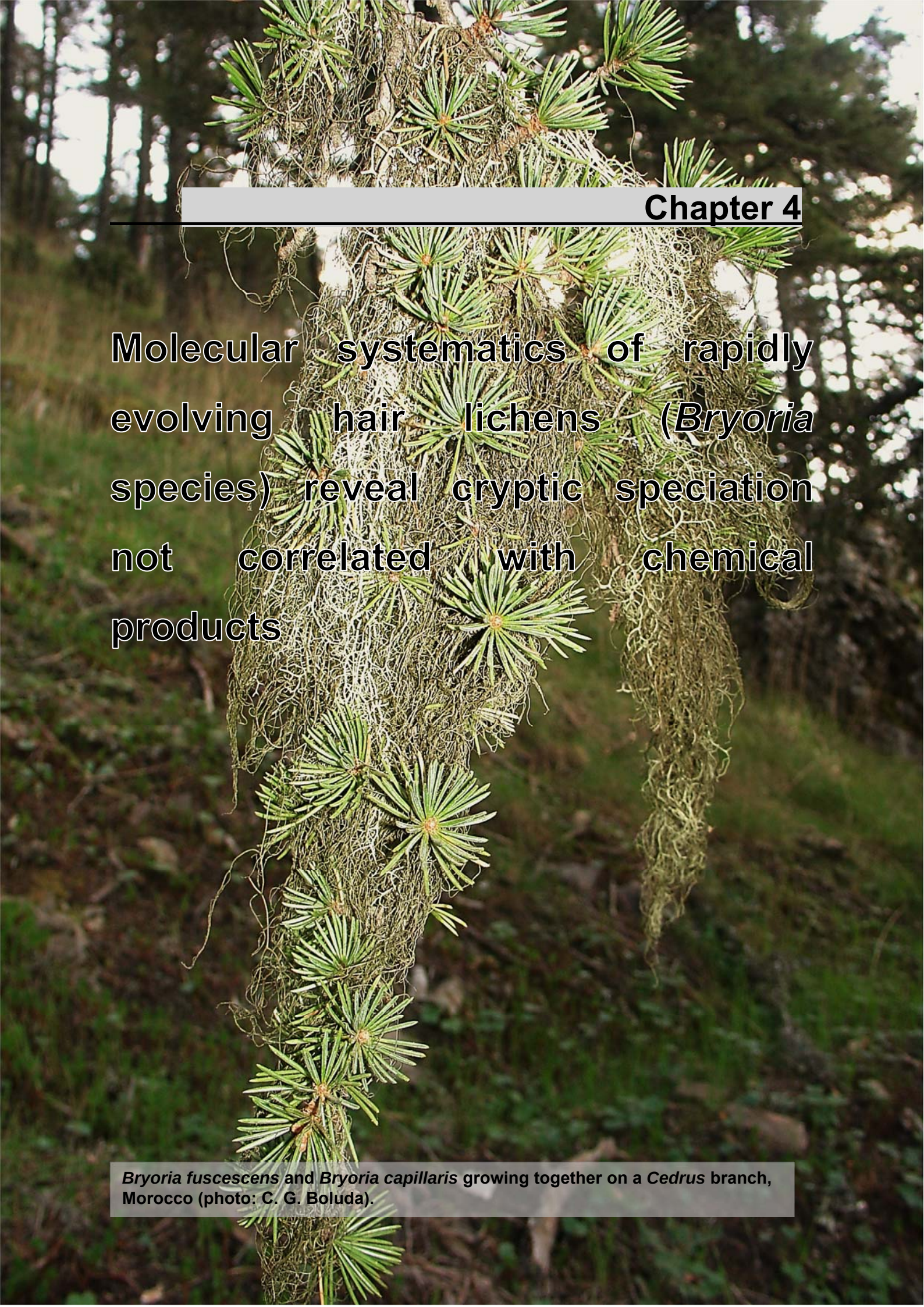
Chapter 4

Molecular systematics of rapidly evolving hair lichens (*Bryoria* species) reveal cryptic speciation not correlated with chemical products



Bryoria fuscescens **and** ***Bryoria capillaris*** **growing together on a** ***Cedrus*** **branch, Morocco (photo: C. G. Boluda).**

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Abstract

Molecular phylogenetic analyses are leading to the discovery of cryptic species within traditional morphospecies in lichen-forming fungi. In contrast, in the case of the traditionally speciose group of hair lichens (*Bryoria* sect. *Implexae*), an integrative taxonomic approach, including morphological, chemical, molecular, and chorological data, leads to the reduction of 11 currently accepted species (separated on the basis of their chemistry and small morphological traits) to just four (three of which can only be distinguished with confidence by molecular methods). We used DNA sequences from the fungal standard barcode locus ITS, IGS, GAPDH, two newly tested loci (FRBi15 and FRBi16), and SSR lengths from 18 microsatellite markers. Chemical products and morphological traits were also recorded. Data were analyzed with Bayesian and maximum likelihood phylogenetic reconstruction, phenogram reconstruction, STRUCTURE Bayesian clustering, principal coordinate analysis, haplotype network, and with species delimitation analyses ABGD, PTP, GMYC, and DISSECT. Additionally, past population demography and node ages have been estimated. The data in all analyses trend to converge into four recently diverged clusters, which are partially cryptic and phenotypically and chemically variable, but show different distributional trends. The three accepted species, all epitypified by sequenced material, are *Bryoria glabra*, *B. fuscescens*, *B. pseudofuscescens*, and *B. kockiana*; in the absence of molecular data, they can be recorded as *Bryoria fuscescens* aggregate. Reasons why some earlier species epithets in the complex are left unepitypified and not taken up are discussed.

Introduction

Chemical characters, mainly the production of polyketides and other compounds, were accorded of major importance in species delimitation in lichen–forming fungi in the 1960s and 1970s, as reviewed elsewhere (Hawksworth 1976, Lumbsch 1998). It is now known that these compounds are formed by the fungal partner in the lichen symbioses (Honegger 1986, Le Pogam *et al.* 2015). Chemical products, generally linked to minor morphological differences, have been used to circumscribe species in the 'hair lichens' of the genus *Bryoria* in *Parmeliaceae* (Brodo & Hawksworth 1977, Myllys *et al.* 2011, Velmala *et al.* 2014). The advent of molecular phylogenetics has enabled such species concepts to be tested, and they have proved wanting in a particular section of the genus, sect. *Implexae* (Myllys *et al.* 2011, Velmala *et al.* 2014, Boluda *et al.* 2015). Among the unknown number of species comprising this section, Velmala *et al.* (2014) considered eleven species and provide DNA molecular data for all of them, viz., *Bryoria capillaris*, *B. friabilis*, *B. fuscescens*, *B. glabra, B. implexa*, *B. inactiva*,

B. kockiana, *B. kuemmerleana*, *B. pikei*, *B. pseudofuscescens*, and *B. vrangiana*. With the exception of *B. glabra*, a genetically well circumscribed taxon, the other species appear to fall into evolutionary units with different, and not concordant, chemical and morphological features. Genetically similar taxa in the complex maintain their phenotypes even when growing in physical contact with one another, and so are growing under identical ecological and environmental conditions (Velmala *et al.* 2014, Boluda *et al.* 2015); they are not, therefore, phenotypic ecological modifications. As a result of consistently observed correlations between chemical products, thallus morphology, and, in some cases ecological preferences (*Myllys et al.* 2016), it is not surprising that these phenotypes have been regarded historically as different species (morphospecies).

While morphological and chemical characters have commonly been used to circumscribe species and provide valuable information for understanding species boundaries, these may not be useful for identifying closely–related species and often fail to accurately characterize species–level diversity in *Parmeliaceae* and lichenized fungi in general (Grube & Kroken 2000, Crespo & Perez–Ortega 2009, Crespo & Lumbsch 2010, Lumbsch & Leavitt 2011). Further, morphological and chemical variations may constitute morpho– or chemotypes of the same species with no molecular differentiation, thus smearing our understanding of species limits. Accurate species delimitation may be obscured by incongruence between morphology and molecular data, or incongruence between gene trees and species trees, and cryptic speciation (Leavitt *et al.* 2016). Molecular sequence data coupled with analytical tools have advanced our knowledge of species recognition (Fujita *et al.* 2012, Leavitt *et al.* 2015). Improved species recognition has important implications for understanding species diversity, speciation, as well as for developing better conservation policies (Bickford *et al.* 2007).

A previous study in one morphospecies of *Bryoria* sect. *Implexae*, *B. fuscescens* agg. (Boluda *et al.* 2015, as *B. fuscescens* s.lat.) revealed specimens sharing the same haplotype inside a single population, but containing different compounds. Additionally, recent extensive field–work across Europe has revealed new combinations of extrolites not previously reported, and specimens sharing characters of different morphospecies. Although some well delimited morphotypes can be recognized (e.g. *Bryoria capillaris*, with predominantly ash–grey color, irregular branching with mainly acute angles and production of barbatolic acid), the data overlap calls into question whether these merit species rank. In order to determine how best to deal with this situation taxonomically, we have combined phenotypic observations with molecular data, including loci not used in the barcoding of fungi (Schoch *et al.* 2012). In particular, we explored whether the generally variable microsatellites or single sequence repeats (SSRs) could assist in establishing genomic relationships among specimens of the

complex, also using different clustering methodologies. This study was also undertaken as experience gained from this group of lichens and it might be expected to inform how other complexes with similar problems could be addressed.

Materials and Methods

Sampling

We used 142 named specimens of 11 *Bryoria* sect. *Implexae* morphospecies from 14 countries, and *B. furcellata* as outgroup (Table 1). These included 91 of the 97 specimens used by Velmala *et al.* (2014) in their revision of *B.* sect. *Implexae* (Table 1, not in bold), and 51 newly obtained sequences shown in bold in Table 1, widely distributed through Europe, the Mediterranean Basin, North America, and Chile. Names used in the analyses follow the species concepts adopted in Velmala *et al.* (2014).

Morphology and chemistry

Morphological and thin layer chromatographic (TLC) analyses of the samples used in Velmala *et al.* (2014, Table 1) are as explained in that work. The newly studied specimens (Table 1, in bold) were examined morphologically under a Nikon SMZ–1000 dissecting microscope, and hand–cut sections studied with a Nikon Eclipse–80i compound microscope equipped with bright field and differential interference contrast (DIC). Habit photographs were taken with a Nikon 105 mm f/2.8D AF Micro–Nikkor Lens coupled to a Nikon D90 camera with daylight. Spot tests (K, C, and PD) and TLC were carried out following Orange *et al.* (2010). Solvent system C (200 ml toluene / 30 ml acetic acid) was used for TLC, with concentrated acetone extracts at 50 ºC spotted onto silica gel 60 F254 aluminium sheets (Merck, Darmstadt, Germany). Spotted sheets were dried for 10 min in an acetic acid atmosphere to maximize resolution. The same lichen fragment used for TLC was also used for DNA extraction to avoid the risk of taking different samples from mixed collections.

DNA dataset

The molecular dataset used comprised DNA sequences and microsatellites or SSRs. DNA extraction was performed with the DNeasy Plant Mini Kit (Qiagen, Barcelona, Spain), following the manufacturer's instructions.

Eighteen fungus microsatellites markers (Bi01, Bi02, Bi03, Bi04, Bi05, Bi06, Bi07, Bi08, Bi09, Bi10, Bi11, Bi12, Bi13, Bi14, Bi15, Bi16, Bi18 and Bi19) were amplified following Nadyeina *et al.* (2014) using fluorescently labelled primers. Fragment lengths were determined on an ABI PRISM® 3130 Genetic Analyser (Life Technologies, Carlsbad, CA, USA). Genotyping was performed using LIZ–500 as internal size standard and GeneMapper v.3.7 (Applied Biosystems, Foster City, CA, USA).

For DNA sequencing, five loci were selected (Table 2), three commonly used as standard markers in fungi (ITS, IGS and GAPDH), used in Velmala *et al.* (2014) in this group, and two microsatellite flanking regions tested for the first time (FRBi15 and FRBi16). Microsatellite flanking regions are variable non–coding DNA fragments that can contain phylogenetical signal (Devkota *et al.* 2014). To explore this possibility, the flanking regions of the 18 microsatellite markers were checked upstream and downstream in the 454 pyrosequencing contigs used for microsatellites searching in Nadyeina *et al.* (2014). The variability of each region was assessed with the number of variable sites in contigs supported by 2 to 16 copies. From the 36 regions (two for each one of the 18 microsatellites), the two most variable were FRBi15 and FRBi16, and these were selected as markers and specific primers designed for them (Table 2).

DNA amplification of the samples used by Velmala *et al.* (2014; Table 1, not in bold), is as explained in that work. For the newly analyzed samples (Table 1, in bold), we used a reaction mixture of 25 µl, containing 12 µl sterile water, 9 µl JumpStartTM REDTaq® ReadyMixTM PCR Reaction Mix (Sigma–Aldrich, St. Louis, MI, USA), 1.25 µl of each primer forward and reverse at 10 µM, and 1.5 µl DNA template. Cycling conditions for ITS, GAPDH, FRBi15 and FRBi16 were 2 min at 94 ºC; 35 cycles of 30 s at 94 ºC, 30 s at 56 ºC; 2 min at 72 ºC; and a final extension of 5 min at 72 ºC. For IGS, the cycling process used was: 2 min at 94 ºC; then 15 cycles of 30 s at 94 ºC, 30 s at 55 ºC (decreasing 1 ºC each cycle down to 40 °C); 2 min at 72 ºC, then 35 cycles of 30 s at 94 ºC, 30 s at 55 ºC; 90 s at 72 ºC; and a final extension of 5 min at 72 ºC. PCR products were checked and quantified on 1 % agarose gel stained with ethidium bromide, and cleaned using Exonuclease I and FastAPTM Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer´s instructions. Sequencing was performed with labeling using BigDye Terminator v.3.1 Kit (Applied Biosystems) as follows: 25 cycles of 20 s at 96 ºC, 5 s at 50 ºC, and 2 min at 60 ºC. Clean–up used BigDye XTerminator Purification Kit (Applied Biosystems) according to the

manufacturer´s instructions. Sequences were obtained in an ABI PRISM® 3130 Genetic Analyser (Life Technologies) and manually adjusted using DNA Workbench (CLC bio, Aarhus, Denmark) and MEGA5 (Tamura *et al.* 2011).

Clustering methodologies

Chemical and morphological clustering

Two presence/absence (1/0) matrices were constructed, one only for the chemical substances detected by TLC, and other including chemistry, morphology, and geography (Table S1). Morphological characters scored comprised: (1) white/dark color; (2) branching angles acute/obtuse/mixed; (3) soralia absent/fissural/tuberculate/both; and (4) pseudocyphellae conspicuous/inconspicuous. For geography, only the regions Old World *vs* New World were taken into account. The R package cluster (Maechler *et al.* 2013) was used to obtain the dissimilarity matrix, and then pvclust package (Suzuki & Shimodaira 2006) was run to obtain a phenogram. Multiscale bootstrap resampling with 10 000 bootstrap (bp) replicates was used to obtain approximately unbiased (au) *p*–values for branch supports. Groups were considered as supported when bp values exceeded 70 or au values exceeded 95.

Phylogenetic tree clustering

Alignments for each locus were performed using MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/, Katoh & Standley 2013) with the G–INS–i alignment algorithm, a '1PAM/K = 2' scoring matrix, with an offset value of 0.1, and the remaining parameters set as default. Alignments were deposited in TreeBASE under accession nos. TB2:S20007 (ITS, IGS and GAPDH), TB2:S20005 (FRBi15), and TB2:S20004 (FRBi16). RDP v.4 (Martin *et al.* 2010) was used to detect potential recombination events in the alignments, through the methods RDP (Martin & Rybicki 2000), GENECONV (Padidam *et al.* 1999), Chimaera (Posada & Crandall 2001), Maxchi (Maynard–Smith 1992), Bootscan (Gibbs *et al.* 2000, Martin *et al.* 2005), SiScan (Weiller 1998, Gibbs *et al.* 2000), PhylPro (Weiller 1998), and 3Seq (Boni *et al.* 2007). Partitionfinder (Lanfear *et al.* 2012) was used to detect possible intra–loci evolutive model variability, resulting in the splitting of the ITS region into ITS1, 5.8S, and ITS2 and coding each codon position separately in GAPDH. Models of DNA sequence evolution for each locus partition were selected with the program jModeltest 2.0 (Darriba *et al.* 2012), using the Akaike information criterion (AIC, Akaike 1974). The best–fit model of evolution obtained was: ITS1 = TIM2, 5.8S = K80, ITS2 = TIM2ef + G, IGS = TrN + I, GAPDH 1st position = TrN + I, GAPDH 2nd position = F81 + I, GAPDH 3th position = TPM3uf, FRBi15 = TPM3uf + I, FRBi16 = TPM3uf + G. To detect possible topological conflicts among loci, the CADM test (Legendre & Lapointe 2004, Campbell *et al.* 2011) was performed using the

function 'CADM.global' implemented in the library 'ape' of R (Paradis *et al.* 2004). Because loci FRBi15 and FRBi16 were not congruent among them and with the remaining loci, three alignments were run to obtain the definitive trees, one for each FRBi region and a concatenated file for ITS, IGS and GAPDH. For the concatenated matrix, specimens with more than one locus missing were rejected. Datasets were analyzed using maximum likelihood (ML) and Bayesian (B/MCMCMC) approaches with gaps treated as missing data.

For maximum likelihood (ML) trees reconstruction, the software RAxML v7.2.8 (Stamatakis 2006) implemented in Cipres Science Gateway (http://qball2.sdsc.edu:7070/portal2/home.action; Miller & al., 2010) was used with the GTRGAMMA model (Stamatakis 2006, Stamatakis *et al.* 2008). Support values were assessed using the 'rapid bootstrapping' option with 1000 replicates. For the Bayesian reconstruction, MrBayes v.3.2.1 (Ronquist & Huelsenbeck 2003) was used. Two simultaneous runs with ten million generations each, starting with a random tree and employing twelve simultaneous chains, were executed. Every 500th tree was saved to a file. Preliminary analysis resulted in an overestimation of branch lengths and to correct this we used the uniform compound Dirichlet prior brlenspr=unconstrained:gammadir (1, 1, 1, 1) (Zamora *et al.* 2015), obtaining rather reasonable branch length estimates. We plotted the log–likelihood scores of sample points against generations using Tracer v.1.5 (Rambaut *et al.* 2014) and determined that stationarity had been achieved when the log–likelihood values of the sample points reached an equilibrium value and ESS values exceeded 200 (Huelsenbeck & Ronquist 2001). Posterior probabilities (PPs) were obtained from the 50 % majority rule consensus of sampled trees after excluding the initial 25 % as burn–in. The phylogenetic tree was drawn with FigTree v.1.4 (Rambaut 2009).

STRUCTURE clustering

The software STRUCTURE v.2.3.4 (Pritchard *et al.* 2000, Falush *et al.* 2003) was run with the microsatellite data matrix. Analysis was computed with 100 000 burn–in generations and 100 000 iterations using a *K* value from 1 to 12 (i.e. the most putative species we may have) and 20 replicates for each *K*. To combine the 20 runs of each *K* value in a single result, CLUMMP v.1.1.2 (Jakobsson & Rosenberg 2007) was used, and visualized replacing the CLUMMP output values in a STRUCTURE output of the same *K*, plotted using the STRUCTURE software. To show the probability of each *K* value, STRUCTURE HARVESTER (Earl & vonHoldt 2012), with the Δ*K* method (Evanno *et al.* 2005) was used, considering the most probable *K* the first one that appears close to 0 in the output graphic.

Principal coordinates analysis clustering

Principal coordinate analysis (PCoA) was carried out using the SSRs length data in GenAlEx 6.5. The results of the three first axes were plotted in a three–axis graph using The Excel 3D Scatter Plot v.2.1, in which the graphic can be moved in 3D to obtain a better understanding of how the plots are distributed in the space. Because the projection of this 3D graph on a paper is necessarily confusing, PCoA results were plotted on two different 2D graphs showing axes 1 and 2, and 1 and 3, respectively.

Haplotype network clustering

Haplotype network reconstruction was carried out using TCS v.1.2.1 (Clement *et al.* 2000) with the concatenated sequences matrix, excluding the outgroup, using gaps as missing data, and a 95 % connection limit. Specimens differing only by missing or ambiguous characters were not counted as haplotypes.

Species delimitation analyses

In order to aid species delimitation in the phylogenetic tree, four computational approaches not requiring prior hypothesis of a putative number of species were used: (1) Automatic Barcode Gap Discovery ABGD (Puillandre *et al.* 2011) based on barcode gaps using genetic distances; (2) Poisson Tree Processes PTP (Zhang *et al.* 2013), based on gene trees; (3) The Generalized Mixed Yule coalescent approach GMYC, which combines a coalescent model of intraspecific branching with a Yule model for interspecific branching (Pons *et al.* 2006, Monaghan *et al.* 2009); and (4) DISSECT (Jones *et al.* 2014) based on multispecies coalescent model for species delimitation. ABGD and PTP were carried out using the online servers placed in http://wwwabi.snv.jussieu.fr/public/abgd/ and http://species.h–its.org respectively. GMYC was analyzed with the gmyc function in the SPLITS package in R (v.2.10, www.cran.r–project.org), employing the single (GMYCsingle) and multiple (GMYCmultiple) threshold methods. Because GMYC needs a strictly ultrametric and bifurcating tree with no zero branch lengths, identical sequences were deleted and ultrametric tree generated using BEAST v.1.8.2 software (Drumond *et al.* 2012), with the evolutionary models explained in the Bayesian phylogenetic reconstruction, a run of 100 million iterations logging every 1 000th iteration and a burn–in of 10 %. ESS values above 200 were ensured using Tracer v.1.6 (Rambaut *et al.* 2014).

DISSECT analysis was implemented in STARBEAST (*BEAST, Drumond *et al.* 2012) using the concatenated DNA matrix after removing identical sequences, and following the instructions of Jones & al. (2014). First we used BEAUti (Drumond *et al.* 2012) to perform the xml file, with every individual encoded as if it was a separate species. Sites, clocks and trees were released as unlinked. Nucleotide substitution models and other parameters (as in the Bayesian analysis, see above), were encoded using BEAUti when possible, or manually

entered. For ITS locus, a substitution rate of 0.033 substitutions per site per million years was introduced (Leavitt *et al.* 2012), setting other loci as estimated with a lognormal relaxed clock. A birth–death–collapse prior that controlled the minimal split heights for the putative resulting species was manually added to the xml file. This prior contained the parameters CollapseHeight (ε) with a value of 0.0001 and CollapseWeight (ω), set as estimated using a Beta distribution with values 10 and 1.5. Selected parameters provide a highest probability density around 4–5 clusters, the most probable number of taxa meriting separation according to other analyses performed for this paper, although this prior is diffuse and different putative more appropriate values can easily be obtained. The xml file was run in BEAST with 250 million of MCMC iterations, sampling every 10 000th iteration. Tracer v.1.6 (Rambaut *et al.* 2014) was used to assess ESS values above 140. The resulting *BEAST species tree output was then treated with SpeciesDelimitationAnalyzer (Jones *et al.* 2014), with a burn–in of 5000 trees (20 % of the total generated), a collapse height of 0.001 (one fraction lower than in the *BEAST analysis) and a simcutoff value of 1 to ignore this parameter, as we expected very similar putative species to emerge. The resulting similarity matrix was plotted with R v.2.15.1 (R Core Team 2014) following the instructions of Jones *et al.* (2014).

Divergence time estimation

Two divergence time estimations were performed, one only with the ITS region and a defined substitution rate, and the other with the concatenated data matrix of ITS, IGS, and GAPDH. The ITS region was not partitioned due to the lack of information about substitution rates for each partition, considering the GTR + G + I model of nucleotide substitution and a substitution rate of 3.30×10^{-9} s·s^{-1}·yr^{-1} for this region as a whole (Leavitt *et al.* 2012). As no previous literature on substitution rates in the IGS and GAPDH of lichen–forming fungi is available, substitution rates of these partitions (as defined in the Bayesian phylogenetic analysis) were set as estimated in the concatenated matrix analysis. A *BEAST v.1.8.2 (Drumond *et al.* 2012) analysis was executed, using a relaxed clock model (uncorrelated lognormal), a birth–death model prior for the node heights and unlinked substitution models, clocks and trees for each of the five loci partitions. Clades G, Ko, NA and WD were selected as potential species, forcing them to keep monophyletic. No calibration points could be used, as no fossils or previous dating of this species complex are available. To avoid stochastic events, two independent analyses were run, each with 200 million generations, sampling each 5000 trees, and discarding the first 10 000 trees (25 %) as burn–in. Tracer v.1.6 (Rambaut *et al.* 2014) was used to ensure ESS parameter values above 115 in the concatenated matrix, and 185 for the ITS analysis. Different priors were tested but no higher ESS values could be obtained, which we suspect was probably due to the very similar sequences, and the uncertain

topology of the backbone connecting the groups Ko, NA, and WD. The two runs performed for each input were merged with logcombiner v.1.8.2 (Drumond *et al.* 2012), and the resulting trees merged in a consensus tree using Tree annotator v.1.8.2 (Drumond *et al.* 2012). Figtree v.1.4 (Rambaut 2009) was used to display the ITS and the concatenated dated species trees.

Demography

Changes in population sizes through time were estimated using the Bayesian skyline analysis (Drumond *et al.* 2005) with BEAST v.1.8.2 (Drumond *et al.* 2012). Only clades Ko, NA and WD, isolated and merged, were studied, as they show a clock–like tree topology and adequate sampling sizes.

As in the divergence time estimation analysis, demography analyses were run using the ITS region without partitioning, with the GTR + G + I model of nucleotide substitution and a substitution rate of 3.30×10^{-9}s·s^{-1}·yr^{-1}, but with a strict molecular clock model (Leavitt *et al.* 2012). Additionally, the same analysis was repeated with the concatenated data matrix with the ITS substitution rate, estimating the other loci rates with a relaxed clock model and using the nucleotide substitution models for IGS and GAPDH explained in the Bayesian phylogenetic reconstruction. Four independent runs for each input were processed with 50 million MCMC generations, sampling parameter values every 5000 generations, using the Bayesian Skyline tree prior model, six discreet changes in population size and with the linear growth option. The two best of the four runs were combined and ESS values checked with Tracer v.1.5 (Rambaut *et al.* 2014), obtaining values usually above 200, with some exceptions with a lower limit of 100. Skyline plots were drawn with Tracer v.1.5.

To support the Bayesian skyline test, a neutrality test to infer if populations are in mutation–drift equilibrium were done. Tajima's D (Tajima 1989) and Fu's F_s (Fu 1997) were calculated with DnaSP v.5.10 (Librado & Rozas 2009). A significantly positive D is interpreted as a diversifying selection or a recent bottleneck, whereas a negative significant D shows purifying selection or a recent expansion. If D is not significantly different from 0, a mutation–drift equilibrium is occurring. Fu's F_s can be interpreted in the same way.

Table 1. Specimen information and GenBank accession numbers of the samples used on this study. Newly obtained sequences in bold.

Taxon	Locality	Source/Voucher	Lab. code	Fig. 3 & 5 code	Chemistry	GenBank accession numbers				
						ITS	IGS	GAPDH	FRBi15	FRBi16
Bryoria capillaris	Canary Islands, Tenerife	Velmala & al. (2014)	S192	87	Ale., Bar.	GQ996289	KJ396490	GQ996261	**KY026810**	**KY002720**
	Canary Islands, Tenerife	MAF–Lich. 20683	L15.15	95	Ale., Bar.	**KY026899**	**KY026945**	**KY026992**	**KY026807**	**KY002718**
	Finland, Etelä–Häme	Velmala & al. (2014)	L141	84	Ale., Bar.	FJ668493	FJ668455	FJ668399	**KY026806**	**KY002697**
	Finland, Etelä–Savo	Velmala & al. (2014)	L211	85	Ale., Bar.	GQ996287	KJ396487	GQ996259	**KY026809**	**KY002711**
	Finland, Uusimaa	Velmala & al. (2014)	S2	88	Ale., Bar	KJ396433	KJ396489	KJ954306	**KY026811**	**KY002729**
	Greece, Peloponese	MAF–Lich. 19670	L06.10	91	Ale. Bar.	**KY026894**	**KY026940**	**KY026987**	**KY026801**	**KY002715**
	Norway, Nord–Trøndelag	Velmala & al. (2014)	L270	86	Bar.	GQ996288	KJ396488	GQ996260	–	–
	Spain, Lérida	MAF–Lich. 19672	L07.15	90	Ale., Bar.	**KY026895**	**KY026941**	**KY026988**	**KY026802**	**KY002716**
	Spain, Madrid	MAF–Lich. 19664	L01.17	89	Ale., Bar.	**KY026893**	**KY026939**	**KY026986**	**KY026800**	**KY002694**
	Spain, Navarra	MAF–Lich. 19674	L08.12	92	Ale. Bar.	**KY026896**	**KY026942**	**KY026989**	**KY026803**	**KY002695**
	Spain, Teruel	MAF–Lich. 20682	L14.02	94	Ale. Bar., Fum., Pso.	**KY026898**	**KY026944**	**KY026991**	**KY026805**	**KY002717**
	Sweden, Västerbotten	MAF–Lich. 19685	L13.03	93	Ale. Bar.	**KY026897**	**KY026943**	**KY026990**	**KY026804**	**KY002696**
	Switzerland, Bivio	MAF–Lich. 20687	L16.21	96	Ale., Bar., Nor., Pso.	**KY026900**	**KY026946**	**KY026993**	**KY026808**	**KY002719**
B. friabilis	Canada	Velmala & al. (2014)	L407	15	Gyr.	KJ396435	KJ396492	KJ954308	**KY026812**	**KY002751**
	Canada, British Columbia	Velmala & al. (2014)	L355	14	Gyr.	KJ396434	KJ396491	KJ954307	–	–
	Canada, British Columbia	MAF–Lich. 20602	fri_02	17	Gyr.	–	**KY083555**	–	–	–
	USA, Alaska	Velmala & al. (2014)	S395a	16	Gyr.	KJ576728	KJ396493	KJ599481	–	–
B. furcellata	Finland, Etelä–Savo	Velmala & al. (2014)	L147	–	Fum.	HQ402722	KJ396494	HQ402627	–	–
B. fuscescens	Canada	Velmala & al. (2014)	S259	71	Fum.	KJ396441	KJ396506	KJ954313	–	–
	Canada, Alberta	Velmala & al. (2014)	S256	70	Fum.	GQ996307	KJ396505	GQ996280	**KY026825**	**KY002702**
	Canada, Alberta	Velmala & al. (2014)	S260a	72	Fum.	KJ396442	KJ396507	KJ954314	–	–
	Canada, Alberta	Velmala & al. (2014)	S261	73	Fum.	KJ396443	KJ396509	KJ954315	–	–
	Canada, Alberta	Velmala & al. (2014)	S267	74	Fum.	KJ576716	KJ396510	KJ599469	–	–
	Canada, Alberta	Velmala & al. (2014)	S272	75	Fum.	KJ576717	KJ396511	KJ599470	–	–
	Canada, Alberta	Velmala & al. (2014)	S369	77	Fum.	KJ396444	KJ396514	KJ954316	–	–
	Canada, Alberta	Velmala & al. (2014)	S379	78	Fum.	KJ396445	KJ396515	KJ954317	–	–
	Canada, Alberta	Velmala & al. (2014)	S380	79	Fum.	KJ396446	KJ396516	KJ954318	–	–
	Canada. Alberta	Velmala & al. (2014)	S274	76	Abs.	GQ996303	KJ396512	GQ996276	–	–
	Canary Islands, Tenerife	MAF–Lich. 20684	L15.21	83	Fum.	**KY026901**	**KY026949**	**KY026996**	**KY026817**	–
	Finland, Ahvenanmaa	Velmala & al. (2014)	L149	61	Fum.	GQ996290	KJ396496	GQ996262	**KY026816**	**KY002698**
	Finland, Etelä–Savo (Epitype)	Velmala & al. (2014)	L139	60	Fum.	KJ396436	KJ396495	KJ954309	**KY026815**	–
	Finland, Koillismaa	Velmala & al. (2014)	S24	69	Fum.	KJ576715	KJ396501	KJ599468	**KY026824**	–
	Finland, Koillismaa	Velmala & al. (2014)	S56	80	Fum.	GQ996291	KJ396502	GQ996263	–	**KY002732**
	Finland, Oulun Pohjanmaa	Velmala & al. (2014)	L189	63	Fum.	GQ996305	KJ396498	GQ996278	**KY026819**	**KY002699**
	Finland, Pohjois–Karjala	Velmala & al. (2014)	S109	67	Fum.	KJ396440	KJ396503	KJ954312	**KY026822**	**KY002701**
	Greenland	Velmala & al. (2014)	L232	65	Abs.	GQ996304	KJ396500	GQ996277	–	**KY002700**
	Norway, Sogn og Fjordane	Velmala & al. (2014)	L224	64	Fum.	KJ396437	KJ396499	KJ954310	**KY026820**	**KY002731**

	Norway, Telemark	Velmala & al. (2014)	L305	66	Fum.	KJ396438	–	–	**KY026821**	–
	Norway, Troms	MAF–Lich. 19681	L12.03	81	Fum.	**KY026902**	**KY026947**	**KY026994**	**KY026813**	–
	Norway, Troms	MAF–Lich. 19682	L12.05	82	Fum.	**KY026903**	**KY026948**	**KY026995**	**KY026814**	**KY002730**
	Russia, Perm Territory	Velmala & al. (2014)	S157	68	Fum.	GQ996306	KJ396504	GQ996279	**KY026823**	**KY002742**
	Sweden, Södermanland	Velmala & al. (2014)	L160	62	Fum.	GQ996300	KJ396497	GQ996272	**KY026818**	–
B. glabra	Chile, IX Region	MAF–Lich. 20595	Bg1	5	Fum.	**KY026904**	**KY026950**	**KY026997**	–	–
	Chile, IX Region	MAF–Lich. 20596	Bg2	6	Fum.	**KY026905**	**KY026951**	**KY026998**	–	**KY002693**
	Chile, IX Region	MAF–Lich. 20597	Bg3	7	Fum.	**KY026906**	**KY026952**	**KY026999**	–	**KY002691**
	Chile, IX Region	MAF–Lich. 20598	Bg4	8	Fum.	**KY026907**	**KY026953**	**KY027000**	–	–
	Chile, IX Region	MAF–Lich. 20599	Bg5	9	Fum.	**KY026908**	**KY026954**	**KY027001**	–	**KY002690**
	Finland, Koillismaa	Velmala & al. (2014)	L186	1	Abs.	FJ668494	FJ668456	FJ668400	–	**KY002688**
	USA, Alaska (Epitype)	*Dillman 11May11:1* (UBC)	L406	2	Abs.	**KY026909**	**KY083556**	**KY026955**	–	**KY002692**
	USA, Alaska	*Dillman 26July11:4* (UBC)	L414	3	Abs.	**KY026910**	**KY083557**	**KY026956**	–	–
	USA, Washington	*Björk 1546* (UBC)	S388	4	Fum.	**KY026911**	–	–	–	**KY002689**
B. implexa	Cyprus, Troodos	MAF–Lich. 19683	L11.15	105	Pso.	**KY026915**	**KY026960**	**KY027005**	**KY026829**	**KY002704**
	Finland, Koillismaa	Velmala & al. (2014)	S22	98	Pso.	GQ996294	KJ396517	GQ996266	**KY026832**	**KY002714**
	Finland, Koillismaa	Velmala & al. (2014)	S36	99	Pso.	KJ576719	KJ396518	KJ599472	**KY026833**	–
	Finland, Koillismaa	Velmala & al. (2014)	S39	100	Pso.	GQ996293	KJ396519	GQ996265	**KY026834**	**KY002733**
	Finland, Koillismaa	Velmala & al. (2014)	S67	101	Pso.	KJ396447	KJ396520	KJ954319	**KY026835**	–
	Greece, Peloponese	MAF–Lich. 19669	L06.05	103	Fum., Pso.	**KY026913**	**KY026958**	**KY027003**	**KY026827**	–
	Morocco, Rif	MAF–Lich. 19679	L10.03	104	Pso.	**KY026914**	**KY026959**	**KY027004**	**KY026828**	**KY002721**
	Russia, Murmansk	Velmala & al. (2014)	S168	97	Pso.	KJ396448	KJ396521	KJ954320	**KY026831**	**KY002705**
	Spain, Madrid	MAF–Lich. 19663	L01.01	102	Pso.	**KY026912**	**KY026957**	**KY027002**	**KY026826**	**KY002703**
	Switzerland, Bivio	MAF–Lich. 20685	L16.15	106	Pso.	**KY026916**	**KY026961**	**KY027006**	**KY026830**	–
B. inactiva	Canada, British Columbia	Velmala & al. (2014)	L206	18	Abs.	GQ996283	KJ396522	GQ996255	–	**KY002760**
	Canada, British Columbia	Velmala & al. (2014)	L323b	19	Abs.	KJ396449	KJ396523	KJ954321	**KY026836**	–
	Canada, British Columbia	Velmala & al. (2014)	L358	21	Abs.	KJ396451	KJ396525	KJ954323	**KY026838**	–
	Canada, British Columbia	Velmala & al. (2014)	S239a	22	Abs.	GQ996284	KJ396526	GQ996256	**KY026839**	–
	Canada, British Columbia	Velmala & al. (2014)	S392a	24	Abs.	KJ396452	KJ396528	KJ954324	–	–
	Canada, British Columbia (Holotype)	Velmala & al. (2014)	L347	20	Abs.	KJ396450	KJ396524	KJ954322	**KY026837**	**KY002761**
	USA, Alaska	Velmala & al. (2014)	S384	23	Abs.	KJ576724	KJ396527	KJ599479	–	–
B. kockiana	USA, Alaska (Holotype)	Velmala & al. (2014)	L394	10	Pso.	KJ396453	KJ396529	KJ954325	**KY026840**	**KY002764**
	USA, Alaska	Velmala & al. (2014)	L396	11	Pso.	KJ396454	KJ396530	KJ954326	**KY026841**	**KY002765**
B. kuemmerleana	Iran, East Azarbaijan	Velmala & al. (2014)	L244a	107	Nor.	GQ996295	KJ396531	GQ996267	**KY026846**	–
	Morocco, Middle Atlas	MAF–Lich. 19677	L09.04	113	Nor.	**KY026918**	**KY026963**	**KY027008**	**KY026843**	**KY002743**
	Morocco, Middle Atlas	MAF–Lich. 19678	L09.07	114	Nor.	**KY026919**	**KY026964**	**KY027009**	**KY026844**	**KY002744**
	Norway, Nord–Trøndelag	Velmala & al. (2014)	L274	108	Nor.	GQ996296	KJ396532	GQ996268	**KY026847**	–
	Norway, Nord–Trøndelag	Velmala & al. (2014)	L275	109	Nor.	KJ396455	KJ396533	KJ954327	–	–
	Russia, Perm Territory	Velmala & al. (2014)	S160	111	Nor.	KJ396456	KJ396535	KJ954328	**KY026849**	–
	Spain, Zamora	MAF–Lich. 19667	L04.03	112	Nor.	**KY026917**	**KY026962**	**KY027007**	**KY026842**	–
	Sweden, Härjedalen	Velmala & al. (2014)	S128	110	Nor.	KJ576720	KJ396534	KJ599473	**KY026848**	**KY002706**
	Switzerland, Bivio	MAF–Lich. 20686	L16.17	115	Nor., Pso.	**KY026920**	–	**KY027010**	**KY026845**	–

B. pikei	Canada, Alberta	Velmala & al. (2014)	S382	34	Ale., Bar.	KJ396466	KJ396547	KJ954338	**KY026864**	**KY002752**
	Canada, Alberta	Velmala & al. (2014)	S383a	35	Ale., Bar.	KJ396467	KJ396548	KJ954339	–	**KY002759**
	Canada, British Columbia	Velmala & al. (2014)	L197	25	Ale., Bar.	KJ396457	KJ396536	KJ954329	–	–
	Canada, British Columbia	Velmala & al. (2014)	L210	26	Ale., Bar.	KJ576714	KJ396539	KJ599467	–	**KY002762**
	Canada, British Columbia	Velmala & al. (2014)	L421	27	Ale.	KJ396462	KJ396543	KJ954334	**KY026850**	**KY002753**
	Canada, British Columbia	Velmala & al. (2014)	L374	28	Ale., Gyr.	KJ396459	KJ396540	KJ954331	**KY026851**	–
	Canada, British Columbia	Velmala & al. (2014)	L376	29	Ale., Gyr.	KJ396460	KJ396541	KJ954332	**KY026852**	**KY002754**
	Canada, British Columbia	Velmala & al. (2014)	L377	30	Ale., Gyr.	KJ396461	KJ396542	KJ954333	**KY026853**	**KY002755**
	Canada, British Columbia	Velmala & al. (2014)	S221	31	Ale., Bar.	KJ396463	KJ396544	KJ954335	–	–
	Canada, British Columbia	Velmala & al. (2014)	S362	32	Ale., Bar.	KJ396464	KJ396545	KJ954336	–	–
	Canada, British Columbia	Velmala & al. (2014)	S368	33	Ale., Bar.	KJ396465	KJ396546	KJ954337	**KY026863**	**KY002756**
	Canada, British Columbia	MAF–Lich. 20601	pik_c	51	Ale.	–	–	–	–	–
	Canada, Nova Scotia	MAF–Lich. 20600	pik_a	49	Ale., Bar.	–	–	–	–	–
	Canada, Prince Edward Island	MAF–Lich. 20603	pik_02	38	Ale., Bar.	**KY026925**	**KY026971**	**KY027014**	**KY026857**	**KY002749**
	Canada, Prince Edward Island	MAF–Lich. 20606	pik_04	39	Ale., Bar.	**KY026926**	**KY026972**	**KY027015**	**KY026858**	**KY002745**
	Canada, Prince Edward Island	MAF–Lich. 20607	pik_05	40	Ale., Bar.	**KY026927**	**KY026973**	**KY027016**	**KY026859**	**KY002727**
	Canada, Prince Edward Island	MAF–Lich. 20609	pik_09	42	Ale., Bar.	–	**KY026975**	–	**KY026861**	**KY002724**
	Canada, Prince Edward Island	MAF–Lich. 20612	pik_10	43	Ale., Bar.	**KY026921**	**KY026965**	–	–	–
	Canada, Prince Edward Island	MAF–Lich. 20610	pik_11	44	Ale., Bar.	**KY026922**	**KY026966**	**KY027011**	**KY026854**	**KY002750**
	Canada, Prince Edward Island	MAF–Lich. 20622	pik_12	45	Ale., Bar.	–	**KY026967**	–	–	–
	Canada, Prince Edward Island	MAF–Lich. 20611	pik_13	46	Ale., Bar.	**KY026923**	**KY026968**	**KY027012**	**KY026855**	**KY002726**
	Canada, Prince Edward Island	MAF–Lich. 20613	pik_14	47	Ale., Bar.	**KY026924**	**KY026969**	**KY027013**	**KY026856**	**KY002728**
	Canada, Prince Edward Island	MAF–Lich. 20614	pik_d	52	Ale., Bar.	–	–	–	–	**KY002725**
	Canada, Quebec	MAF–Lich. 20608	pik_07	41	Ale., Bar.	–	**KY026974**	**KY027017**	**KY026860**	**KY002723**
	Canada, Quebec	MAF–Lich. 20605	pik_15	48	Ale., Bar.	–	**KY026970**	–	–	**KY002741**
	Canada, Quebec	MAF–Lich. 20604	pik_b	50	Ale., Bar.	**KY026928**	–	**KY027018**	**KY026862**	**KY002748**
	USA, Alaska	Velmala & al. (2014)	S390	36	Ale., Bar.	KJ396468	KJ396549	KJ954340	–	–
	USA, Oregon	Velmala & al. (2014)	S394	37	Ale., Bar.	KJ576727	KJ396550	KJ599480	–	–
B. pseudofuscescens	Canada, British Columbia (Epitype)	Velmala & al. (2014)	S222	53	Nor.	KJ396469	KJ396551	KJ954341	**KY026865**	**KY002757**
	Canada, British Columbia	Velmala & al. (2014)	S232	54	Nor.	KJ396470	KJ396552	KJ954342	**KY026866**	–
	Canada, British Columbia	Velmala & al. (2014)	S370	55	Nor.	KJ396471	KJ396553	KJ954343	–	–
	Canada, British Columbia	Velmala & al. (2014)	S371	56	Nor.	KJ396472	KJ396554	KJ954344	–	–
	USA, Alaska	Velmala & al. (2014)	S377	57	Nor.	KJ396473	KJ396555	KJ954345	–	–
	USA, Alaska	Velmala & al. (2014)	S386	58	Nor.	KJ576725	KJ396556	KJ599478	–	**KY002707**
	U.A, Alaska	Velmala & al. (2014)	S387	59	Nor.	KJ576726	KJ396557	KJ599477	**KY026867**	**KY002758**
***B.* sp.**	USA, Alaska	Velmala & al. (2014)	L395	12	Abs.	KJ396486	KJ396581	KJ954358	**KY026869**	**KY002766**
	USA, Alaska	Velmala & al. (2014)	L392	13	Abs.	KJ396485	KJ396580	KJ954357	**KY026868**	**KY002763**
B. vrangiana	Canada, Alberta	Velmala & al. (2014)	S385	124	Fum.	KJ396484	KJ396578	KJ954356	**KY026886**	**KY002739**
	Finland, Kainuu	Velmala & al. (2014)	S341b	123	Abs.	KJ396483	KJ396577	KJ954355	**KY026885**	–
	Finland, Kainuu	Velmala & al. (2014)	S72	131	Abs.	KJ396481	KJ396573	KJ954353	**KY026892**	**KY002740**
	Finland, Koillismaa	Velmala & al. (2014)	S10	119	Gyr.	GQ996297	KJ396564	GQ996269	**KY026882**	–
	Finland, Koillismaa	Velmala & al. (2014)	S42	125	Gyr.	KJ396478	KJ396566	KJ954350	**KY026887**	**KY002710**
	Finland, Koillismaa	Velmala & al. (2014)	S45	126	Abs.	GQ996302	KJ396568	GQ996275	**KY026888**	–
	Finland, Koillismaa	Velmala & al. (2014)	S57	127	Fum.	KJ576722	KJ396570	KJ599475	–	–
	Finland, Koillismaa	Velmala & al. (2014)	S59	128	Fum.	KJ396480	KJ396571	KJ954352	**KY026889**	–

Finland, Oulun Pohjanmaa	Velmala & al. (2014)	S196a	122	Abs.	KJ396482	KJ396576	KJ954354	**KY026884**	–
Finland, Uusimaa	Velmala & al. (2014)	S6	130	Fum.	KJ396477	KJ396563	KJ954349	**KY026890**	–
Finland, Varsinais–Suomi	Velmala & al. (2014)	S62	129	Gyr.	KJ576721	KJ396572	KJ599474	**KY026891**	–
Italy, Sicily	MAF–Lich. 19668	L05.17	135	Fum.	**KY026931**	**KY026978**	**KY027021**	**KY026872**	**KY002709**
Morocco, Rif	MAF–Lich. 19680	L10.13	140	Abs.	**KY026936**	**KY026983**	**KY027026**	**KY026877**	**KY002722**
Norway, Nord–Trøndelag	Velmala & al. (2014)	L272	116	Gyr.	GQ996299	KJ396558	GQ996271	**KY026880**	**KY002747**
Norway, Nord–Trøndelag	Velmala & al. (2014)	L273	117	Abs.	KJ396474	KJ396559	KJ954346	–	–
Norway, Nord–Trøndelag	Velmala & al. (2014)	L300	118	Abs.	GQ996301	KJ396562	GQ996274	**KY026881**	**KY002746**
Norway, Oppland	Velmala & al. (2014)	L307	132	Gyr.	KJ396439	–	KJ954311	–	–
Norway, Troms	MAF–Lich. 19684	L12.11	141	Fum., Gyr.	**KY026937**	**KY026984**	**KY027027**	**KY026878**	**KY002738**
Russia, Perm Territory	Velmala & al. (2014)	S164	120	Abs.	GQ996285	KJ396574	GQ996257	**KY026883**	**KY002712**
Russia, Perm Territory	Velmala & al. (2014)	S166	121	Fum.	GQ996308	KJ396575	GQ996273	–	**KY002713**
Spain, Asturias	MAF–Lich. 19666	L03.07	134	Fum.	**KY026930**	**KY026977**	**KY027020**	**KY026871**	**KY002708**
Spain, Cáceres	MAF–Lich. 19665	L02.20	133	Fum.	**KY026929**	**KY026976**	**KY027019**	**KY026870**	**KY002734**
Spain, Lérida	MAF–Lich. 19671	L07.03	136	Fum.	**KY026932**	**KY026979**	**KY027022**	**KY026873**	**KY002735**
Spain, Lérida	MAF–Lich. 19673	L07.19	137	Abs.	**KY026933**	**KY026980**	**KY027023**	**KY026874**	**KY002736**
Spain, Navarra	MAF–Lich. 19675	L08.19	138	Abs.	**KY026934**	**KY026981**	**KY027024**	**KY026875**	**KY002737**
Spain, Navarra	MAF–Lich. 19676	L08.20	139	Fum.	**KY026935**	**KY026982**	**KY027025**	**KY026876**	–
Sweden, Västerbotten	MAF–Lich. 19686	L13.12	142	Gyr.	**KY026938**	**KY026985**	**KY027028**	**KY026879**	–

Abs. = No substances detected, Ale. = Alectorialic acid, Bar. = Barbatolic acid, Fum. = Fumarprotocetraric acid, Gyr. = Gyrophoric acid, Nor. = Norstictic acid, Pso. = Psoromic acid.

Table 2. Primer information.

Marker	Description	Primer forward (5'–3')	Source	Primer reverse (5'–3')	Source
ITS	Internal transcribed spacers of the nuclear rDNA including the 5.8S region	ITS1–F: CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns (1993)	ITS4: TCCTCCGCTTATTGATATGC	White *et al.* (1990)
IGS	Intergenic spacer of the nuclear rDNA	IGS12b: AGTCTGTGGATTAGTGGCCG	Printzen & Ekman (2002)	SSU72R: TTGCTTAAACTTAGACATG	Gargas & Taylor (1992)
GAPDH	Glyceraldehyde 3–phosphate dehydrogenase gene partial sequence	Gpd1–LM: ATTGGCCGCATCGTCTTCCGCAA	Myllys *et al.* (2002)	Gpd2–LM: CCACTCGTTGTCGTACCA	Myllys *et al.* (2002)
FRBi15	Flanking region of *Bryoria* sect. *Implexae* microsatellite marker 15	FRBi15f: GTCATAAGGGTATCAATCC	This paper	FRBi15r: TGAAAAGGTTTGGTGACTC	This paper
FRBI16	Flanking region of *Bryoria* sect. *Implexae* microsatellite marker 16	FRBi16f: CGAGGTTTCAGGAAAGGGAA	This paper	FRBi16r: AGGAAGTGATGTCGAGGT	This paper

Results

Morphological and chemical clustering

The wide geographical range of the studied collections revealed a combination of characters not previously reported in the complex, especially those from the previously less–studied Mediterranean region specimens. Specimens with intermediate morphologies amongst traditionally accepted species were recognized, and the application of species names according to the current taxonomy was ambiguous. TLC results revealed seven different extrolites: alectorialic, barbatolic, fumarprotocetraric, gyrophoric, norstictic, and psoromic acids, and atranorin, and sometimes also related substances such as chloroatranorin, protocetraric or connorstictic acids. Atranorin, a typical accessory substance in the genus *Bryoria*, was not used in the posterior analyses because it appears in trace amounts in many samples, and is often difficult to unequivocally discern if it is present or absent.

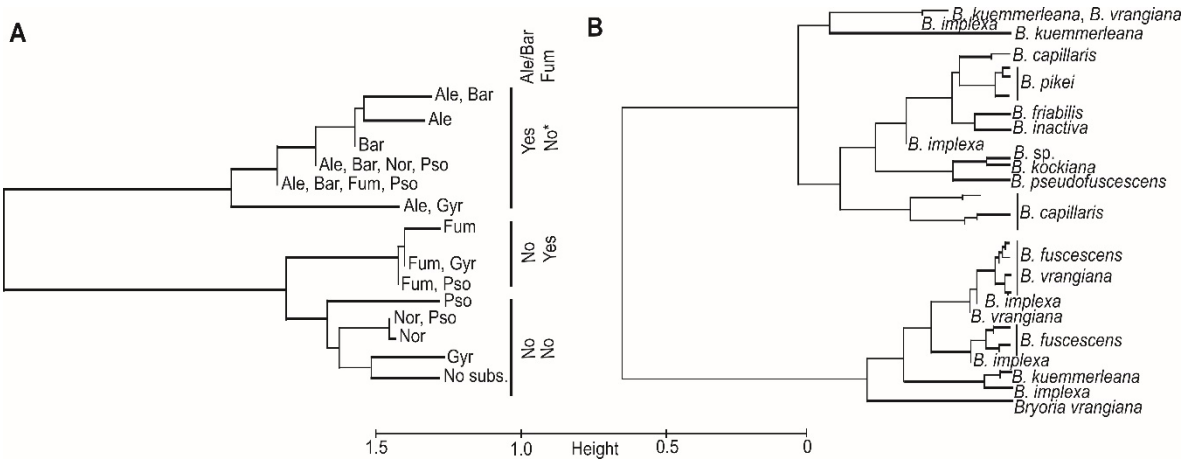


Fig. 1. Phenograms based on a presence/absence distance matrix from: Left. Extrolite composition; Right. Extrolite composition, geographical, and morphological data. — Bold branches represent supported clades (bootstrap ≥ 70 %, approximately unbiased ≥ 95 %). — Ale = Alectorialic acid; Bar = Barbatolic acid; Fum = Fumarprotocetraric acid; Gyr = Gyrophoric acid; No subs. = No substances detected; Nor = Norstictic acid; Pso = Psoromic acid; * = Except the specimen named *Bryoria capillaris* L14.02.

The chemical presence/absence matrix resulted in a phenogram in Fig. 1 Left showing 14 chemotypes. Specimens with as many as four substances, not previously reported in the literature, act as linking chemotypes, making the definition of clearly isolated chemical groups impractical. Rather than the combination of compounds, we considered better to characterize possible groups according to the presence or absence of particular compounds. Specimens

with benzyldepsides (i.e. alectorialic and barbatolic acids), were clearly separated from specimens in which these compounds were not detected; these substances have traditionally been used to separate *Bryoria capillaris* and *B. pikei* from other species in the section. Specimens without benzyldepsides but with detected depsidones clustered in two well supported groups, one with fumarprotocetraric acid as main substance and the other without it.

The analysis of combined morphological, geographical, and chemical characters resulted in a phenogram in Fig. 1 Right. Only terminal branches were supported, including few monophyletic morphospecies, although not isolated from others. Phenotypic groups could not be recognized, and none taxon separated. The ambiguity is largely caused by phenotypical intermediate specimens mainly from the Mediterranean region, but also by the overlapping characters amongst the traditional morphospecies, as is the presence/absence of soralia, pseudocyphellae, characteristic extrolites and thallus color.

Phylogenetic tree clustering

Due to the topological conflict between loci, three DNA matrices were used to generate three phylogenetic trees: (1) concatenated regions ITS, IGS and GAPDH with 134 individuals, 1774 bp and 83 parsimony informative (Pi) sites; (2) the FRBi15 with 93 individuals, 569 bp and 44 Pi sites; and (3) the FRBi16 with 80 individuals, 632 bp and 160 Pi sites.

No evidence of possible recombination events was detected in the concatenated matrix (ITS, IGS and GAPDH). The resulting tree (Fig. 8) has four well supported main clades, G (Glabra, yellow color), Ko (Kockiana, magenta), NA (North American, blue), and WD (Widely Distributed, red + green + brown). Clade G includes material of *Bryoria glabra* only, an isolated taxon sister to the other three clades, which were not unequivocally resolved. Clade Ko includes material under the name of *Bryoria kockiana* and two unidentified *Bryoria* specimens, all specimens are from Alaska (USA). Clade NA comprises the previously recognized North American morphospecies group (as 'North American endemic species' in Velmala *et al.* 2014) named *Bryoria friabilis*, *B. inactiva*, *B. pikei*, and *B. pseudofuscescens*; these species were mixed in the tree, but the group as a whole was resolved as monophyletic. The WD clade, includes specimens widely distributed but mainly from Europe (as 'European and globally distributed species' group in Velmala *et al.* 2014) under the names *Bryoria capillaris*, *B. fuscescens* (syn. *B. chalybeiformis* and *B. lanestris*), *B. implexa*, *B. kuemmerleana*, and *B. vrangiana*. None of these taxa were resolved as monophyletic.

The phylogram produced using the FRBi15 and FRBi16 markers formed a different tree topology, not congruent with that from the preceding concatenated data set. In the FRBi15 tree

reconstruction (Fig. 2 Left), *Bryoria glabra* was not represented due to excess of primer specificity in the PCR reaction, and the tree could not be rooted. Several well supported groups

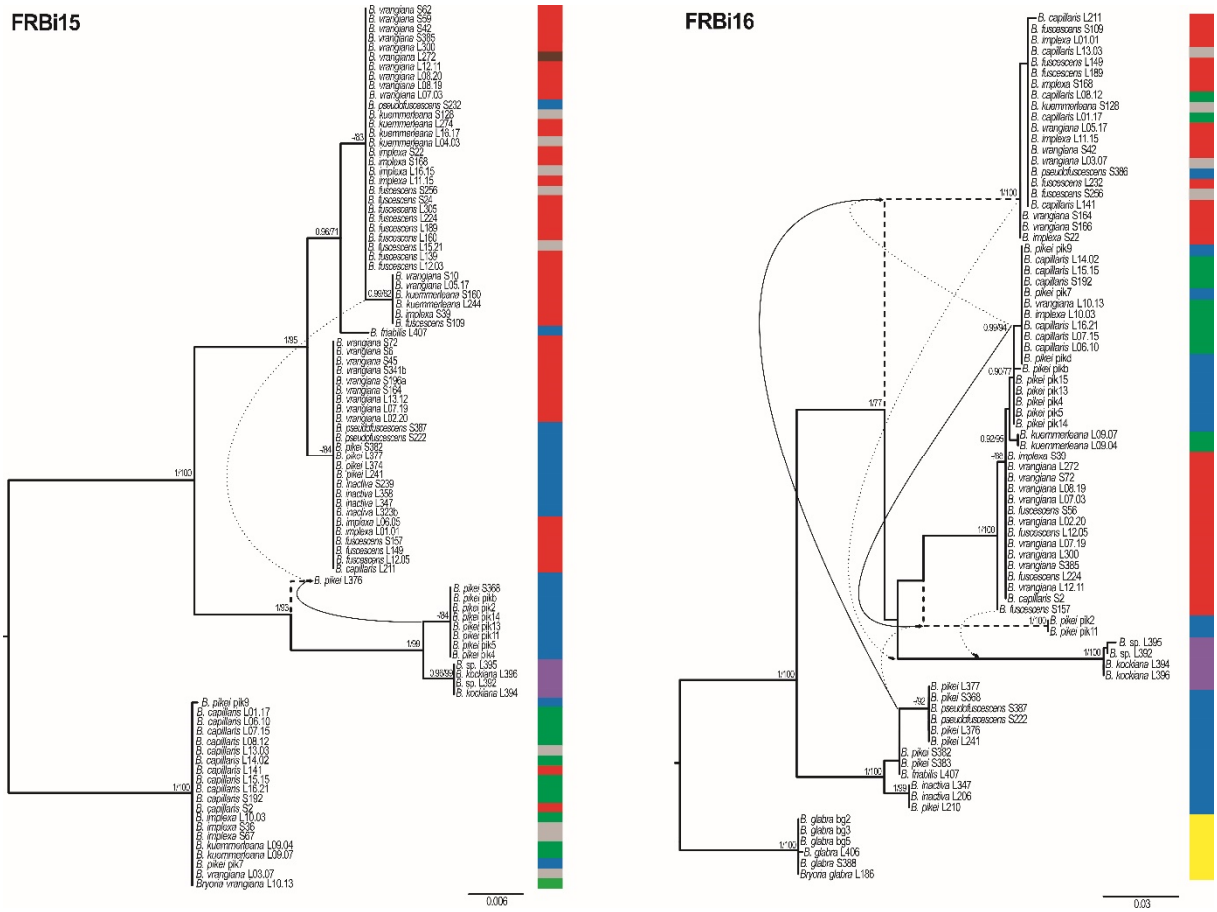


Fig. 2. Phylogenetic relationships in *Bryoria* sect. *Implexae* based on FRBi15 and FRBi16 markers. Tree topology depicts the result of the Bayesian Markov chain Monte Carlo (B/MCMC) analysis. Posterior probabilities and bootstrap analysis for the supported nodes (≥ 0.95 and ≥ 70 %) are indicated at the main nodes. Lines connecting clades indicate putative recombination events, with main parents (continuous lines) and minor parents (discontinuous lines). Because the clade insertion in the trees is influenced by the recombination, clades with recombination are depicted with a discontinuous branch line. Note that clades with recombination appear as sister or close to the main parent, but tending to be deviated towards the minor parent. — Colored bar corresponds to the SSR genepool from Fig. 4, with specimens intermediate among two or more genepools in grey.

were produced, but did not follow any evident geographic, morphologic, chemical, or microsatellite pattern. *Bryoria pikei* L376 has a sequence with a putative recombination fragment with the *B. vrangiana* S10 clade in c. 50 % of the total length. This insertion placed the specimen out of the main parental group appearing as its sister. Marker FRBi16 (Fig. 2 Right) also produced a well–structured tree with supported nodes, but with a different topology from that obtained using other loci, and incongruent with that based on phenotypic, geographic, or microsatellite data. However, in this case *Bryoria glabra* appeared as a well isolated taxon

and served to root the tree. An analysis to detect putative recombination events gave many possibly results amongst the clades Ko, NA and, WD. If it is not caused by artifacts, the analysis suggests these events were real and a reticulate evolution could be occurring. In both trees of Fig. 2, clade Ko was monophyletic, but did not appear clearly isolated from the others, and could have resulted from a recombination event among them.

STRUCTURE clustering

From the eighteen microsatellite markers from Nadyeina *et al.* (2014), only the nine that showed more than 95 % successful amplification across the samples were used (number of haplotypes in brackets; Table S2): Bi01 (17), Bi03 (6), Bi04 (8), Bi05 (5), Bi10 (8), Bi11 (9), Bi12 (12), Bi14 (6), and Bi19 (5). We allowed a maximum of three missing–data markers per specimen, value reached in seven specimens. Eleven named species were present in the input of the Bayesian clustering performed with STRUCTURE, but 11 clusters were not evident in the output (Fig. 3). STRUCTURE was allowed to run up to K = 12, but from K = 6 the clustering process started to be uninformative. The likelihood results of the ΔK analysis (Evanno *et al.* 2005) indicated three as the most probable number of clusters (likelihood = –1232, ΔK = 2.2; Fig. S1), the clades G, NA and WD (Fig. 3, K = 3). Clade Ko, which appeared isolated in the concatenated phylogenetic tree (Fig. 8), could not be accepted as distinct under this hypothesis. However, *Bryoria glabra* was not clearly isolated in STRUCTURE. This could be attributable to clustering algorithms being influenced by unbalanced sampling sizes, which could be also masking clade Ko appearing isolated at K = 6. From K = 4 to K = 6 the new groups appear mainly inside the WD clade, showing that collected samples from Europe are much more diverse than those from North America. Indeed, the NA clade was not split into subgroups even at K = 10. This strongly supports rejection of currently used species concepts in the complex. Apart from *Bryoria glabra*, no other morphospecies form a distinct cluster even at high K values. The results support a four species rather than a 12 species hypothesis. Specimen 49 (under *B. pikei*, Fig. 3 marked with an asterisk), probably comes to a misidentification with the phenotypically similar *B. capillaris*; however, as sequence amplification of this specimen failed, we cannot determine if this mismatch was due to a morphospecies misidentification or a DNA contamination.

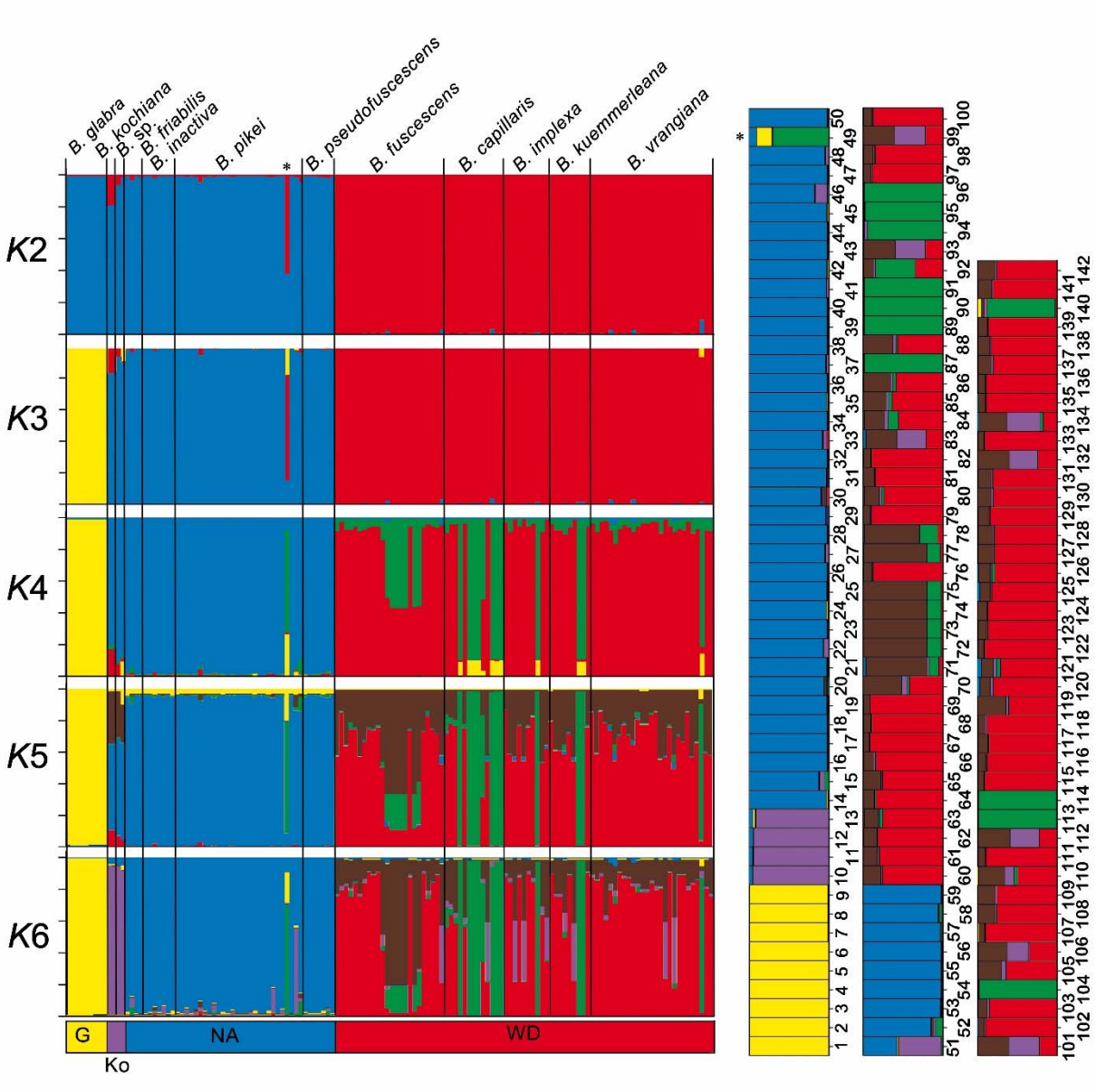


Fig. 3. Bayesian inference of population structuring using STRUCTURE.v.2.3.4 (Pritchard *et al.* 2000, Falush *et al.* 2003) and 9 microsatellite loci. Left. Results from the hypothesis of 2 to 6 clusters. Vertical bars represent specimen assignment probability into a genetic cluster depicted in colors. Morphospecies names given to the specimens appear at the top. Right. Detailed columns of the K = 6 hypothesis, the numbers representing the specimens shown in Table 1 for a better understanding of the clusters component of each individual. — G = Glabra clade, Ko = Kockiana clade, NA = North American clade, WD = Wide Distributed clade, * = *Bryoria pikei* specimen 49

Principal coordinates analysis (PCoA)

The results of the PCoA analysis (Fig. 4) represent the three–dimensional output in two different graphics, comparing axis 1 against 2, and 1 against 3. The information percentage of each axis was 44.47 %, 15.06 %, and 14.44 %, respectively. Clade G (Fig. 4 yellow) appeared isolated, and clade Ko (Fig. 4 magenta) admixed with NA clade (Fig. 4 blue), forming both a single cluster. Clade WD was isolated from the others, but divided into two clusters, one corresponding to the red and brown groups in the *K* = 6 STRUCTURE output (Fig. 3), and one for the green group. Apart from *Bryoria glabra*, no currently accepted species formed a defined group. In brief, four reasonably isolated clusters could be distinguished, corresponding to the groups G, Ko + NA, WDr (Wide Distributed, Fig. 4 red) and WDg (Wide Distributed, Fig. 4 green).

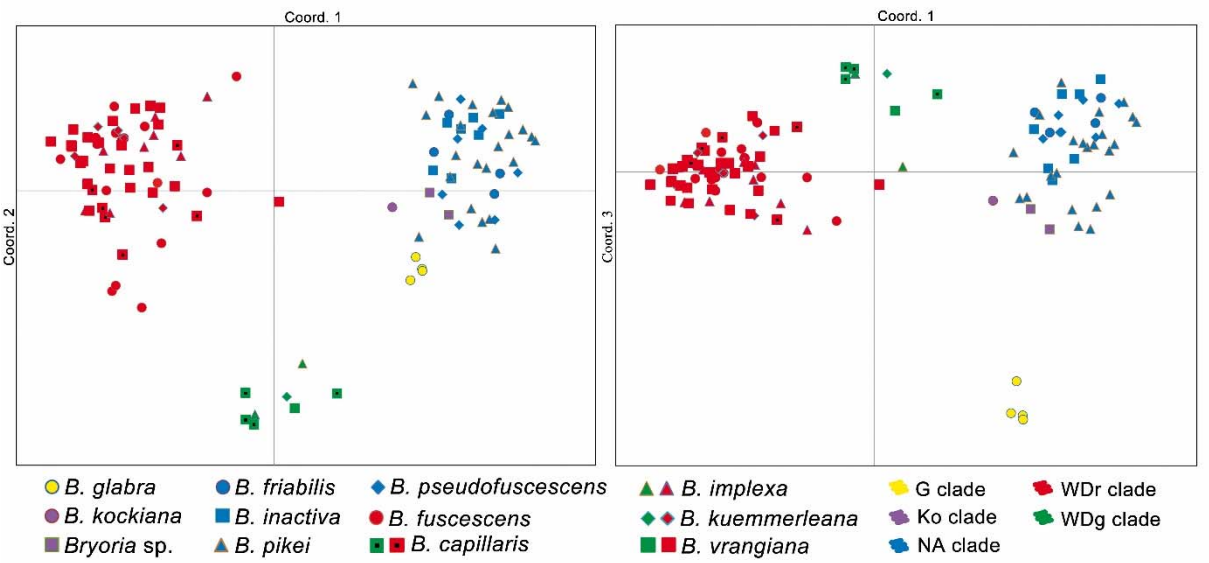


Fig. 4. Principal Coordinate Analysis of microsatellite data. Traditional species names (shape and colors) and STRUCTURE clusters (colors) for each specimen, represented in the three main coordinates. Note that Ko clade appears not isolated from NA clade in any coordinate axis. — G = Glabra clade, Ko = Kockiana clade, NA = North American clade, WDr = Widely Distributed red clade, WDg = Widely Distributed green clade.

Haplotype network clustering

The haplotype network of the concatenated data matrix, with gaps as missing data, produced 39 haplotypes. *Bryoria glabra* specimens (Fig. 5 yellow) formed two haplotypes not connected with other members of the network, indicating genetic isolation of this species. One of the haplotypes was composed exclusively of South American specimens, whereas the other contained European, North American, and South American specimens. Clade Ko (Fig. 5

magenta) fell into two haplotypes, the one including specimens with psoromic acid and identified as *Bryoria kockiana*, and the other with unidentified samples in which no substances were detected. This group was connected to the NA clade (Fig. 5 blue) by a long branch with 13 mutation steeps. The NA clade was separated by nine mutations steps from the WDg and WDr clades (Fig. 5 green and red, respectively). The WDg cluster did not appear as isolated from the WDr cluster. When two networks are obtained from a single analysis with a 95 % parsimony connection limit, each network might be considered a different species (Hart & Sunday 2007). In the case of connections with several mutation steeps, taxon delimitation is more ambiguous, but in our analysis, due to the extent of the isolation of each subnetwork and that this topology is coincident with other data, clades G, Ko, NA and WD are revealed as putative taxa, without any distinction between WDr and WDg.

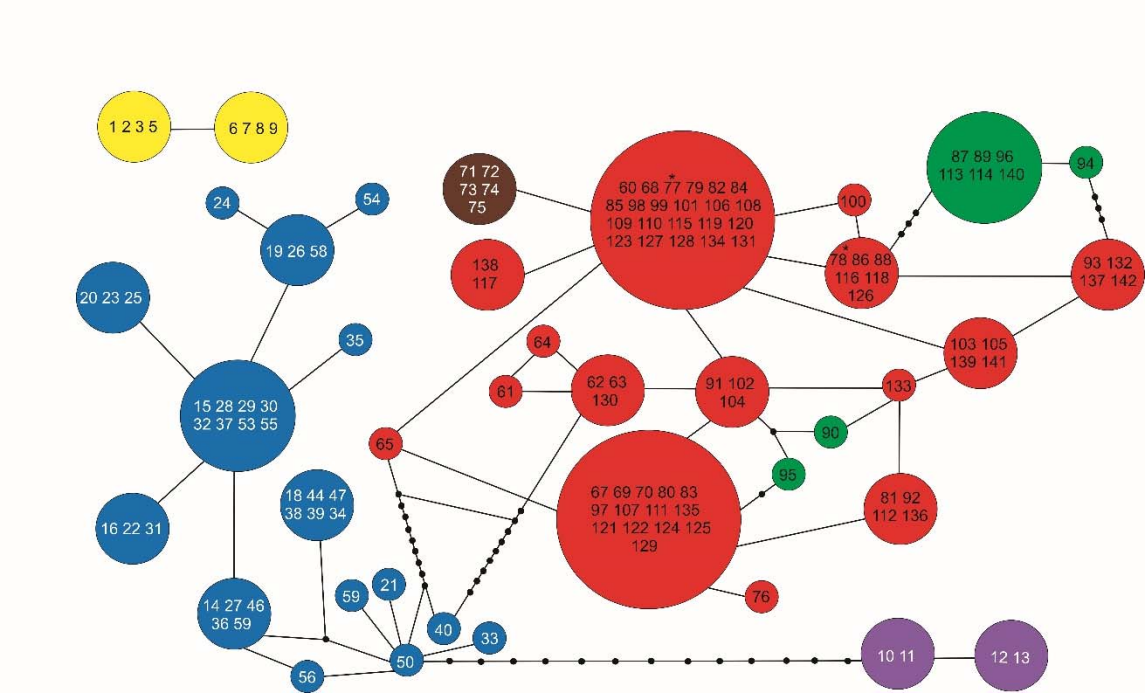


Fig. 5. Haplotype network of the DNA sequence concatenated matrix containing the loci ITS, IGS and GAPDH with gaps as missing data and 95 % of connection limit. Numbers represent the specimens showed in Table 1 and colors depict the STRUCTURE microsatellites genepool (Fig. 3). Connecting line length do not depict the genetic distance. Each line represents a single mutation as well as each black small circles. Circle size is related with the number of containing specimens. — * = WDb (Wide Distributed brown cluster) specimens.

Table 3. Species delimitation analysis results for ITS, IGS, GAPDH and the concatenated data matrix. Brackets indicate groups predicted as conspecific. — G = Glabra clade, Ko = Kockiana clade, NA = North American clade, WD = Wide Distributed clade, WDr = Wide Distributed red clade, WDg = Wide Distributed green clade, pik5 = Specimen *Bryoria pikei* 5.

Method	ITS	IGS	GAPDH	Concatenated
ABGD	2 = G + (Ko, NA, WD)	2 = G + (Ko, NA, WD)	4 = G + Ko + NA + WD	4 = G + Ko + NA + WD
PTP	2 = G + (Ko, NA, WD)	2 = G + (Ko, NA, WD)	4 = G + Ko + NA + WD	5 = G + Ko + NA + WDr + WDg
GMYCs	4 = G + (Ko, NA, WDg) + WDr + WDr	3 = G + (Ko, WD) + NA	4 = G + Ko + NA + WD	6 = G + Ko + NA + pik5 + WDr + WDg
GMYCm	4 = G + (Ko, NA, WDg) + WDr + WDr	4 = G + (Ko, WD) + NA1 + NA2	4 = G + Ko + NA + WD	5 = G + Ko + NA + WDr + WDg
DISSECT	–	–	–	5 = G + Ko + NA + pik5 + WD

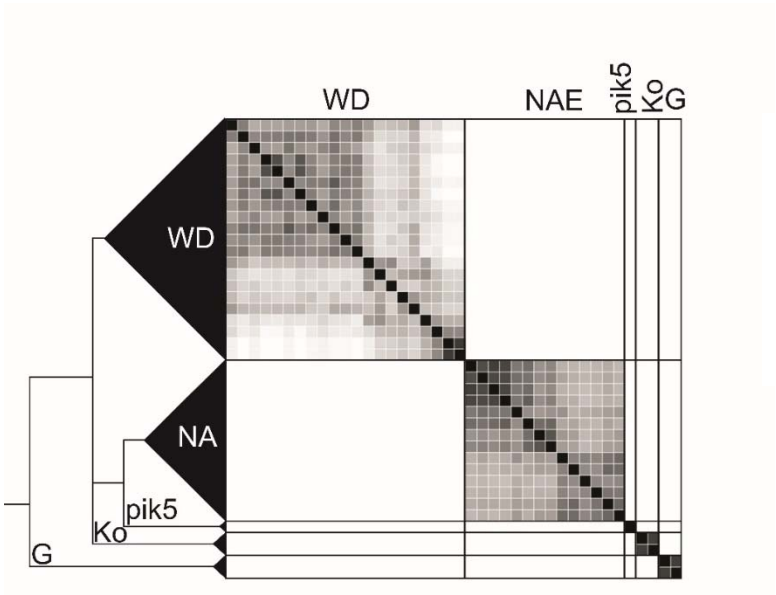


Fig. 6. Similarity matrix from DISSECT analysis performed after clone correction. Squares represent posterior probability (white = 0, black = 1) of pairs of specimens to belong to the same species. Resulting major groups are delimited with lines that indicate the clade on the collapsed phylogenetic tree.

Species delimitation software

The ABGD, PTP, GMYC, and DISSECT programs (Table 3) uses different algorithms, and consequently can predict different number of putative species. The genetic distance method (ABGD) gave the smallest number, whereas the coalescence methods (especially GMYC) the largest. Analyses also revealed the contribution of each locus to the postulated species delimitated, GAPDH being the most informative and constant marker.

DISSECT is a recent powerful method to detect species previously used just one time in fungi Mark *et al.* (2016). This analysis (Fig. 6) predicted five clusters, the G, Ko and NA clades appearing as well separated apart from one specimen in the NA cluster (*Bryoria pikei* 5) which formed an independent group as also predicted by $GMYC_{simple}$. The WD clade was also well defined, and although two internal greyish square–groups are depicted, they do not correspond exactly to WDr and WDg.

Node dates and demographic history

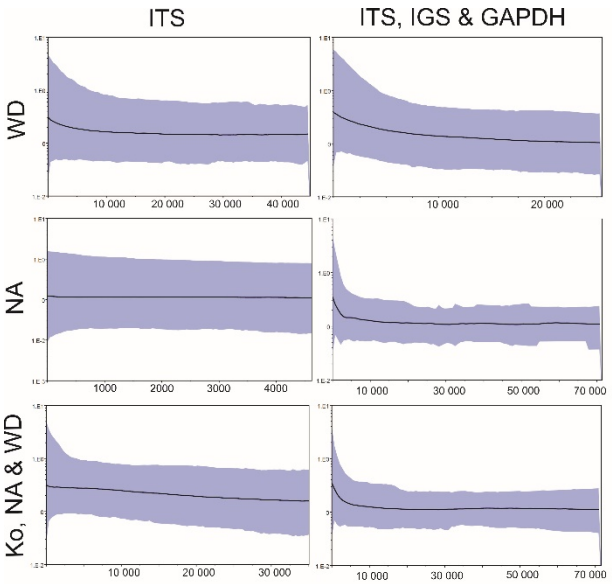
The calibrated maximum clade credibility chronogram for the concatenated data matrix is represented as Fig. 7. Because only the ITS mutation rate is known, a second chronogram was prepared using this locus alone. Results from this analysis have to be treated with caution, as the species tree is not strictly clock–like (*Bryoria glabra* has a shorter branch), and the ITS mutation rate has been taken from a different lichen–forming genus, *Melanohalea*, in the same family (*Parmeliaceae*). Both analyses produced similar values, and the divergence of *Bryoria glabra* lineage was estimated at 0.69 Mya (95 % HPD = 0.35—1.08) from the concatenated matrix analysis, and 0.65 Mya (95 % HPD = 0.22—1.14) from the ITS data. The Ko, NA and WD split was calculated at 0.10 Ma (95 % HPD = 0.03—0.22) from the concatenated matrix and 0.06 Mya (95 % HPD = 0.02—0.15) from the ITS data.

Bayesian Skyline Plots (Fig. 7 Left) show a recent population increase in the NA and WD clades. However, the sequences contain few informative mutations and the deepest coalescence was reached in a few thousand years, with no population changes detectable further back from this period. Genealogies of most plant and animal species coalesce 2.58—0.01 Mya (Grant 2015), but in some of our results coalescence reached earlier. The sampling of genes with low levels of polymorphism could avoid the successful reconstruction of population history. A flat graphic is generally interpreted as population stability, but can be also due to a lack of detection power by small sample sizes. Moreover, a small rise in the curve near the present can be produced to the random sampling of the MCMC haplotype trees (Grant 2015) and, consequently, this result has to be treated with caution. Our results seem to indicate that the contemporary sequences do not bear imprints of ancient population history, but rather

recent population diversification. The loss of information can be produced by bottlenecks (strong reduction of a population size), but also to local extinctions and subsequent recolonization (constant population size at metapopulation level).

Test of neutrality (Fig. 7 Right) are commonly used to support inferences from Bayesian Skyline Plots. As indicated by non–significant Tajima's D and F_s results, all sampled groups are in mutation–drift equilibrium. There is only one exception, the GAPDH locus of the NA clade, with a significant negative D value (Fig. 7 bold). This indicates a recent expansion or purifying selection, as seen in the concatenated Bayesian Skyline analysis, but other loci did not support this hypothesis.

Markers ITS, IGS and GAPDH indicate population stability over the immediate past for clades NA and WD, with putative very recent small population expansions. As a consequence of the low variability of the loci, and the putative loss of demographic signals, this hypothesis cannot be confirmed.



	ITS			IGS			GAPDH			Concatenated		
	h	Fs	D	h	Fs	D	h	Fs	D	h	Fs	D
WD	5	0.66 P>0.1	-0.16 P>0.1	4	-0.88 P>0.05	-0.54 P>0.1	1	not polymorphism		10	-1.42 P>0.1	0.37 P>0.1
NA	4	-2.18 P>0.1	-1.27 P>0.1	3	-1.61 P>0.1	-1.13 P>0.1	3	**-0.39 P>0.02**	**-2.18 P>0.01**	10	-3.78 P>0.1	-1.02 P>0.1
Ko, NA & WD	9	-2.07 P>0.1	-0.52 P>0.1	5	-2.20 P>0.1	-0.97 P>0.1	4	7.67 P>0.1	0.61 P>0.1	18	-0.78 P>0.1	-0.28 P>0.1

Fig. 7. Left. Bayesian Skyline Plots for each clade predicted by the ITS marker and the concatenated loci matrix. The x axis of each graph represent time in years, whereas y–axis represents the value for the log of the effective population size as relative changes, because generation times in Bryoria species are unknown. Gray shadows indicate upper and lower 95 % credible intervals. Right. results from neutrality tests for each marker and clade, indicating in bold a statistically significant deviation from the neutrality. — h = number of haplotypes, Fs = Fu's Fs, D = Tajima's D.

Integrated assessment of datasets

Depending on the analysis, different numbers of putative species groups were suggested, ranging from three to six (Table 4). Our analysis, confirmed that the combination of characters traditionally used for species recognition in this group was inadequate. GAPDH, despite its low variability, was the most informative of the markers tested, and the only one separating and supporting clades Ko, NA, and WD (Table 3). ITS, one of the most used loci for DNA barcoding in lichen–forming fungi, did not unambiguously distinguish those three clades. The new markers FRBi15 and FRBi16, despite their higher variability, showed inconclusive results and putative recombination events. The microsatellite data (Fig. 2) reflected internal variability unrevealed by DNA sequences, showing that WD cluster was much more diverse than NA, which had a particularly low diversity.

Table 4. Summary of the number of putative species suggested by the different methods used for each data set.

Method	Data	Figure / reference	Number of putative species
Traditional concept	DNA sequences and phenotypic	Velmala *et al*. (2014)	12
Chemical	Phenotypic	Fig.1 Left	c. 4
Morpho-chemical	Phenotypic	Fig.1 Right	Not conclusive
Phylogeny	DNA sequences of ITS, IGS and GAPDH	Fig. 8	4 = G + Ko + NA + WD
Phylogeny	DNA sequences of FRBi15	Fig. 2	Not conclusive
Phylogeny	DNA sequences of FRBi16	Fig. 2	Not conclusive
STRUCTURE	Microsatellites	Fig. 3	5 = G + Ko + NA + WDr + WDg
PCoA	Microsatellites	Fig. 4	4 = G + (Ko, NA) + WDr + WDg
Haplotype Network	DNA sequences of ITS, IGS and GAPDH	Fig. 5	4 = G + Ko + NA + WD
ABGD	DNA sequences of ITS, IGS and GAPDH	Table 3	4 = G + Ko + NA + WD
PTP	DNA sequences of ITS, IGS and GAPDH	Table 3	5 = G + Ko + NA + WDr + WDg
GMYCs	DNA sequences of ITS, IGS and GAPDH	Table 3	6 = G + Ko + NA + pik5 + WDr + WDg
GMYCm	DNA sequences of ITS, IGS and GAPDH	Table 3	5 = G + Ko + NA + WDr + WDg
DISSECT	DNA sequences of ITS, IGS and GAPDH	Fig. 7	5 = G + Ko + NA + pik5 + WD

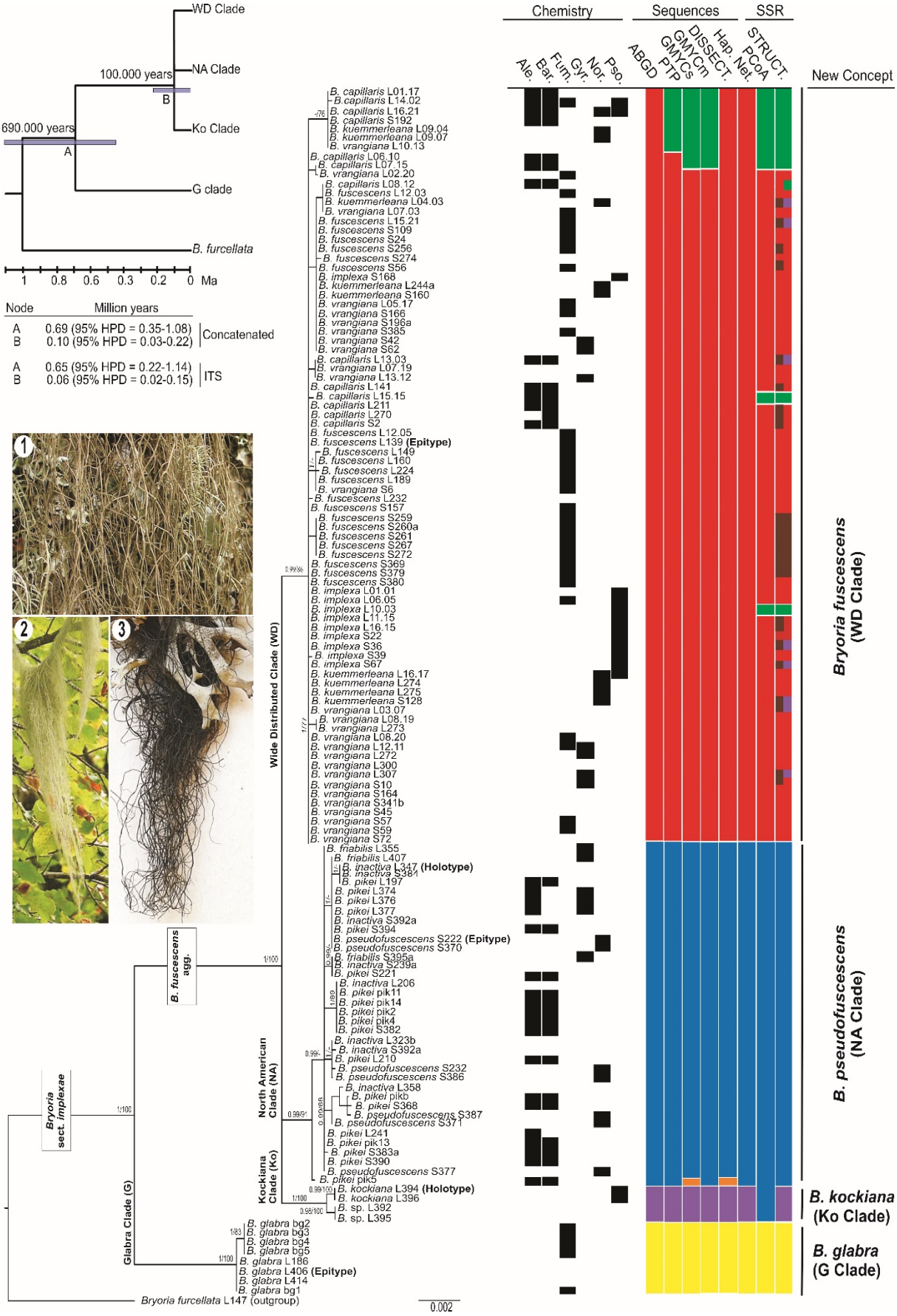

WD Clade
NA Clade
Ko Clade
G clade
B. furcellata
100.000 years
690.000 years
A
B
1 0.8 0.6 0.3 0.2 0 Ma
Node Million years
A 0.69 (95% HPD = 0.35-1.08)
B 0.10 (95% HPD = 0.03-0.22)
Concatenated
A 0.65 (95% HPD = 0.22-1.14)
B 0.06 (95% HPD = 0.02-0.15)
ITS
Chemistry
Sequences
SSR
Ale.
Bat.
Fum.
Cyr.
Nor.
Pso.
ABGD
PTP
GMYCs
GMYCm
DISSECT.
Hap. Net.
PCoA
STRUCT.
New Concept
Wide Distributed Clade (WD)
B. fuscescens agg.
North American Clade (NA)
Kockiana Clade (Ko)
Glabra Clade (G)
Bryoria sect. implexae
Bryoria fuscescens (WD Clade)
B. pseudofuscescens (NA Clade)
B. kockiana (Ko Clade)
B. glabra (G Clade)
Bryoria furcellata L147 (outgroup)
0.002

Fig. 8. Integrated assessment of results. Tree topology depicts the result of the Bayesian Markov chain Monte Carlo (B/MCMC) analysis. Posterior probabilities and bootstrap analysis for the supported nodes (≥ 0.95 and ≥ 70 %) are indicated at the main nodes. For each specimen is showed the extrolites detected, and the putative number of clusters predicted by the different methodologies. The small tree depicts the results from the node datation analysis. — 1 = *Bryoria implexa* morphotype (Spain, Asturias, 2013, Boluda, MAF–Lich. 20749), 2 = *B. capillaris* morphotype (Spain, Navarra, 2013, Boluda & Villagra, MAF–Lich. 20748), 3 = *B. fuscescens* morphotype (Morocco, Ifrane, 2013, Boluda, MAF–Lich. 20751). — Ale. = Alectorialic acid, Bar. = Barbatolic acid, Fum. = Fumarprotocetraric acid, G = Glabra clade, Gyr. = Gyrophoric acid, Hap. Net. = Haplotype Network, HPD = Highest Posterior Density, Ko = Kockiana clade, NA = North American clade, Nor. = Norstictic acid, PCoA = Principal Coordinates Analysis, Pso. = Psoromic acid, STRUCT. = Structure, WD = Wide Distributed clade

Taxonomy

Bryoria sect. ***Implexae*** (Gyeln.) Brodo & D. Hawksw., Opera Bot. 42: 114. 1977.

Basionym. Bryopogon sect. *Implexae* Gyeln., Feddes Repert. 38: 223. 1935.

Type species. Bryoria implexa (Hoffm.) Brodo & D. Hawksw. 1977. ≡ *Usnea* [unranked] *implexa* Hoffm. 1796 (= *Bryoria fuscescens* (Gyeln.) Brodo & D. Hawksw. 1977, but see below).

Species with fruticose, hair–like, subpendent to mainly pendent thallus, lateral spinules or spinulose branches absent, whitish gray to brown or black. Angles between branches variable, acute to obtuse or even rounded. Pseudocyphellae absent or present, then frequently inconspicuous, ± fusiform, concolorous or whitish. Soralia absent or present, tuberculate or fissural, white to dark. Isidia or isidioid spinules absent. Apothecia mainly absent, if present, usually afunctional. Chemistry also varied, with no detectable or with one or a combination of key substances among alectorialic, barbatolic, connorstictic, fumarprotocetraric, gyrophoric, norstictic, protocetraric, psoromic and salazinic (probable) acids, atranorin and chloroatranorin. Photobiont *Trebouxia 'hypogimniae'* (Lindgren et al. 2014). Widely distributed.

Notes — Most species included in Brodo & Hawksworth (1977) under *Bryoria* sect. *Implexae* were transferred to other sections in Myllys et al. (2011). In the light of our results, but see the Discussion, *Bryoria* sect. *Implexae* includes the following four species. Comments on particular morphological or chemical traits that may be helpful for distinguish these four taxa are given under each species. Nevertheless, a wide overlap of all cited characteristics exists among different specimens, leading us to consider them cryptic. The included species names are epitypified here in order to fix their identities at the molecular level. This is because no DNA sequences can be easily obtained from the old type materials associated with most species names, so those types cannot be critically identified for purposes of the precise application of

the name to a taxon (McNeill et al. 2012, Art. 9.8). As a result, we recommend using the collective name *Bryoria fuscescens* agg. for any specimen lacking molecular data, but see also Discussion.

Bryoria fuscescens (Gyeln.) Brodo & D. Hawksw., Opera Bot. 42: 83. 1977.

Basionym. Alectoria fuscescens Gyeln., Nytt Mag. Natur. 70: 55. 1932, nom. cons.

Type specimens. FINLAND, Tavastia austr., Hollola, ad truncos *Pini* locis aprici oribus in silva, Sept. 1882, *J.P. Norrlin* (Norrlin, Herb. Lich. Fenn. No. 46) (BP 33947 lectotype designated by Hawksworth 1972: 217). — FINLAND, Etelä–Savo, Savitaipale, NW of Mustapää, 600 m, 61,1721 ºN, 27,6900 ºE, 2005, *L. Myllys 464* (H 9209715 (L139) epitype designated here).

Nomenclature — A large number of species rank names may belong to this group, but these have not been epitypified with sequenced material. These include *Bryoria capillaris* (Ach.) Brodo & D. Hawksw. 1977 (≡ *Parmelia jubata* [var.] *capillaris* Ach. 1803, *Alectoria capillaris* (Ach.) Cromb. 1871), *B. chalybeiformis* (L.) Brodo & D. Hawksw. 1977 (≡ *Lichen chalybeiformis* L. 1753 nom. rej. against *B. fuscescens*), *B. implexa* (Hoffm.) Brodo & D. Hawksw. 1977 (≡ *Usnea* [unranked] *implexa* Hoffm. 1796, *U. implexa* (Hoffm.) Hoffm. 1799), *B. kuemmerleana* (Gyeln.) Brodo & D. Hawksw. 1977 (≡ *Alectoria kuemmerleana* Gyeln. 1932), *B. lanestris* (Ach.) Brodo & D. Hawksw. 1977 (≡ *Alectoria jubata* [var.]. *lanestris* Ach. 1810, *A. lanestris* (Ach.) Gyeln. 1932), *B. subcana* (Nyl. ex Stizenb.) Brodo & D. Hawksw. 1977 (≡ *Alectoria prolixa* var. *subcana* Nyl. ex Stizenb. 1892 nom. rej. against *B. fuscescens* and *A. subcana* (Nyl. ex Stizenb.) Gyeln. 1931), *B. vrangiana* (Gyeln.) Brodo & D. Hawksw. 1977 (≡ *Alectoria vrangiana* Gyeln. 1932). *Bryoria austromontana* P.M. Jørg. & D.J. Galloway 1983 described from Oceania (Jørgensen & Galloway 1983) may also belong here.

The earliest species rank epithets amongst these are *chalybeiformis* dating from 1753 (*Lichen chalybeiformis*), and *implexa* dating from 1799 (*Usnea implexa*). The former has been rejected against *Bryoria fuscescens*, but not against other species names apart from *B. subcana* (Hawksworth & Jørgensen 2013). A proposal to add *Usnea implexa* to the names against which *Alectoria fuscescens* is conserved is being prepared separately, and accordingly the name *B. fuscescens* is used here following Rec. 14A of the ICN (McNeill et al. 2012). We decided not to take up these or other competing names by epitypification here as the name *B. fuscescens* is very well–known and already conserved over two species names in the complex. To take up any other species names would be confusing as their traditional circumscriptions are linked to particular morphologies or chemistries.

Notes — *Bryoria fuscescens* is highly variable in morphology and chemistry, and some of the analyzed specimens develop soralia. Further, atranorin, which is not normally detectable in the other three species accepted here, is frequently detected in concentrated extracts from both sorediate and esorediate morphs.

Distribution — Widely distributed, known from Europe, Asia, North America, and Africa. There are also reports under various names treated here as probable synonyms from Antarctica, New Zealand, and South America,

Bryoria glabra (Motyka) Brodo & D. Hawksw., Opera Bot. 42: 86. 1977.

Basionym. Alectoria glabra Motyka, Fragm. Florist. Geobot. 6: 448. 1960.

Type specimens. USA, Washington, Olympic Peninsula, Clallam Co., Hurricane Ridge, 5800 ft, on trunk of *Abies lasiocarpa*, 24 July 1950, *B.I. Brown & W.C. Muenscher 129* (US holotype). — USA, Alaska, Mainland, Chugach National Forest, Paradise Valley between the Bucher and Gilkey Glaciers, 11 May 2011, *K.L. Dillman 11May11:1* (UBC (L406) epitype designated here).

Notes — Morphologically and chemically difficult to separate from any other species in *Bryoria* sect. *Implexae*. Distinguishing features in well–developed specimens of this species are, the brownish thallus with a regular branching pattern, generally with obtuse and rounded angles between the branches, and broad oval and usually white soralia. Only fumarprotocetraric and protocetraric acids have been detected, and these are characteristically produced in the soralia.

The Alaskan specimen is selected as epitype here as sequences are available from all loci. Material from Washington state only has data on the ITS locus. The Alaskan specimen comes from the Chugach National Forest, in the south of Alaska approaching the Olympic Peninsula.

Distribution — Known only from northern Europe (Scandinavia), and North and South America. In North America it is most abundant in the Pacific North–West.

Bryoria kockiana Velmala, Myllys & Goward, Ann. Bot. Fenn. 51: 361. 2014.

Type specimen. USA, Alaska, Fairbanks, North Star Borough, 26 July 2011, *D. Nossov 20019–1* (UBC (L394) holotype).

Notes — Few specimens of this species have been studied. It is characterized by the absence of a whitish grey tone in the thallus, the lack of soralia, and the greyish to brown

branches with conspicuous, white to concolorous, broad, elongate–fusiform, sometimes slightly raised pseudocyphellae, and without substances or with psoromic acid.

Distribution — Known only from Alaska (USA) and British Columbia (Canada), on conifer branches.

Bryoria pseudofuscescens (Gyeln.) Brodo & D. Hawksw., Opera Bot. 42: 127. 1977.

Basionym. *Alectoria pseudofuscescens* Gyeln., Ann. Hist.–Nat. Mus. Natl. Hung. 28: 283. 1934 and, Rev. Bryol. Lichenol. 7: 51. 1934.

= *Bryoria inactiva* Goward, Velmala & Myllys, Ann. Bot. Fenn. 51: 360. 2014.

Type specimens. USA, Oregon, Benton County, Corvallis, on old apple trees, Dec. 1931, *F.P. Sipe 669* (BP 33958 holotype of *Alectoria pseudofuscescens*). — CANADA, British Columbia, 25 September 2006, *T. Goward 07–02–0011* (UBC (S222) epitype selected here). — CANADA, British Columbia, Clearwater Valley, 0.5 km S of Philip Creek, 'Edgewood West', 715 m, 9 Nov. 2011, *T. Goward 11–61* (UBC (L347) holotype of *Bryoria inactiva*).

Nomenclature — A number of species rank names may belong to this group, but these have not been epitypified with sequenced material. These include *Bryoria friabilis* Brodo & D. Hawksw. 1977, *B. pikei* Brodo & D. Hawksw. 1977, and *B. salazinica* Brodo & D. Hawksw. 1977. All these epithets, however, are much later than *pseudofuscescens* and so would not have priority over that name.

Notes — Characterized by the absence of soralia and detectable atranorin.

Distribution — Only known from North America, growing on bark, branches, rock or soil.

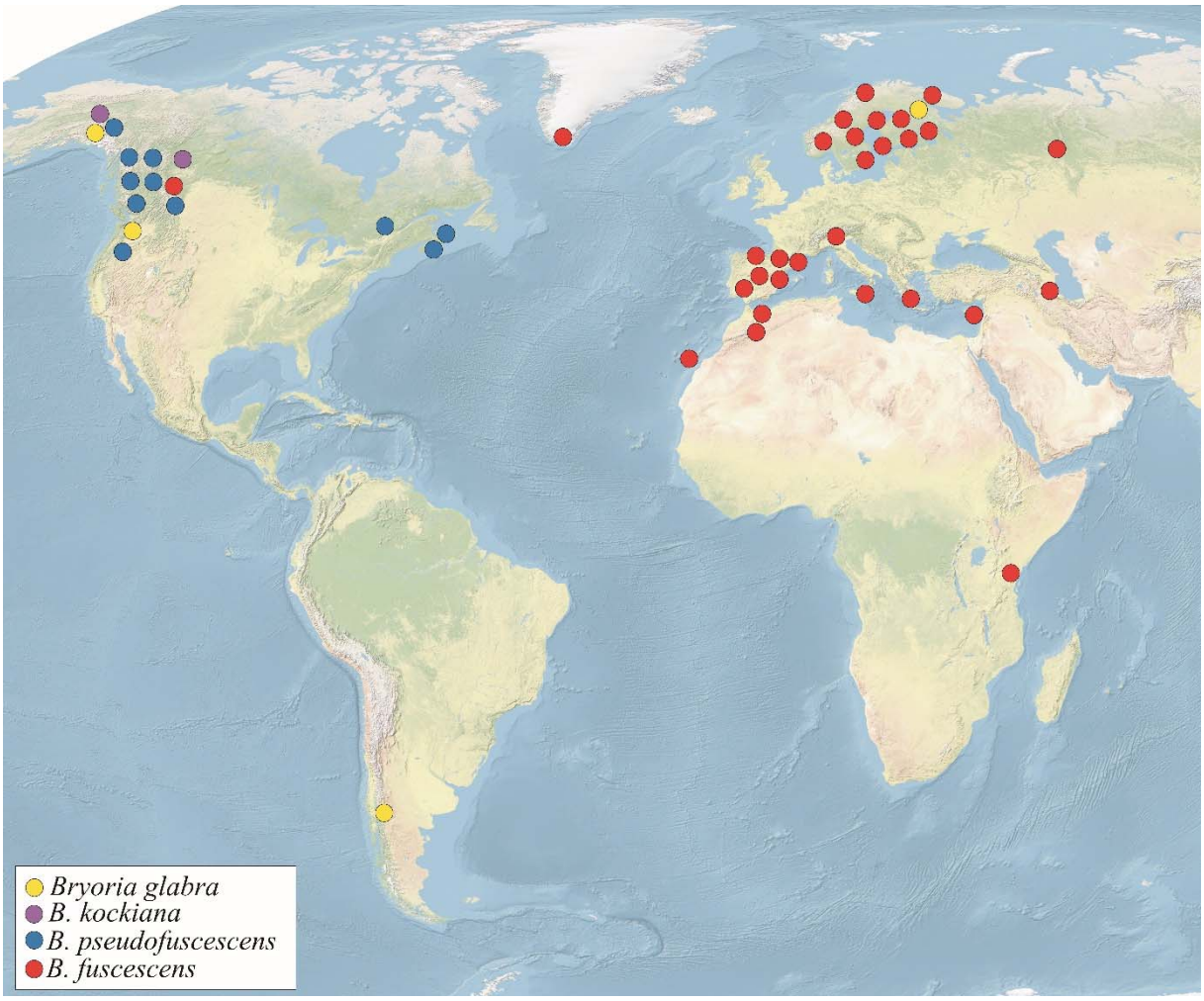


Fig. 9. Distribution of examined specimens. Two samples of geographical interest, not analyzed in this study, has been added: *Bryoria kockiana* (Paratype: Canada, British Columbia, 1982, Goward 82–1040, UBC, cf. Velmala *et al*. 2014) and *Bryoria fuscescens* (Tanzania, Kilimanjaro, 2016, Boluda & Kitara, MAF–Lich. 20750). — Basemap source: U. S. National Park Service (NPS) Natural Earth physical map.

Discussion

Integrative taxonomy has been demonstrated to be a powerful tool to resolve complex taxonomic groups. The methodologies test if phenotypic characters used are artificial, and help to find the most taxonomically informative ones. Traditional species concepts in *Bryoria* sect. *Implexae* have been based primarily on well characterized northern European and North American specimens, but demarcations break down when more variable southern European specimens are studied. When phenotypic data are compared with molecular results, it is evident that the traditionally used characters for species delimitation are unreliable. DNA sequences appear to provide more objective group delimitations, but caution is needed when

not previously tested loci are being used in phylogeny as not every marker's evolutionary history might be congruent with species evolutionary history. Although with the present data it is difficult to discern if the incongruences are due to hybridization or incomplete lineage sorting. The last phenomenon is characteristic of evolving lineages with high diversification rates and large effective population sizes, as is presumed for our study group (Saag *et al.* 2014, Blanco–Pastor *et al.* 2012). In addition, no detectable hybridization is expected for these organisms because of their predominant or entirely asexual reproductive strategy. Nevertheless, we cannot exclude that the incongruences are caused by the analysis of different paralogs of FRBi15 and FRBi16, amplified with the new primers. This seems improbable, because no paralogs have been detected for the SSRs themselves.

Microsatellites are widely used in population studies of many organisms due to their high variability. In species complexes with diffuse genetic barriers, microsatellite data can be used to improve DNA sequence resolution (Lumley & Sperling 2011). In the present case, the microsatellites revealed a similar pattern to that obtained from DNA sequences, and show the WD clade to be much more diverse than NA, although this could be due to an unbalanced number of environments being sampled in each clade. Phenotypic, DNA sequence, and microsatellites data can be processed through a great variety of clustering software to avoid subjective species delimitation.

Our results have a tendency to converge into four clusters, G, Ko, NA and WD (Table 4 and Fig. 8). It is well known that some species delimitation analyses, such as GMYC, PTP, STRUCTURE, etc., can overestimate the number of taxa meriting formal recognition, particularly when sampling is uneven or in species with strong intra–specific genetic structure (Altermann *et al.* 2014, Modica *et al.* 2014). ABGD is often considered as a rather conservative method, less prone to species overestimation and particularly less sensitive to unbalanced sampling, although since this method only detects discontinuities in DNA sequence variation (Puillandre *et al.* 2011), it is expected to also fail for species with strong intra–specific structure, e.g., species with populations containing exclusive haplotypes. A common mistake is considering that all of these species delimitation methods directly give a number of 'species' as output, while they are just providing a number of reasonably discrete groups ('evolving lineages') that should be evaluated in order to be considered as species; i.e. species delimitation analyses provide a number of 'putative species'. The evaluation of those putative species is crucial and strongly dependent on the expertise of the specialist on the study group. This process can be objectivized by analyzing some of the classical properties defining a species (sometimes treated as 'species concepts'), such as phenotypical differentiation, niche specificity, reproductive isolation, etc. (de Queiroz 2007).

Our study group shows a broad phenotypic (morphological + chemical) overlap, and therefore, phenotypic characters are not the best data to differentiate species here, or at least morphological data suggest that a single variable species is involved. The G clade is perhaps the only exception, as the morphology and chemistry of the studied samples is relatively constant, being often possible to characterize this taxon by phenotypic data, despite some overlap with other samples still exists. Niche specificity was not specifically investigated, but some samples of the WD group have a similar geographical origin than others in the NA group; we expect that a more intense sampling will reveal more specimens of the WD group growing together with specimens of the NA group.

Reproductive isolation is difficult to address in lichens, since their reproductive biology is not fully understood yet. In addition, the study group comprises lichen–forming fungi that mostly or exclusively show an asexual reproduction, entangling even more its reproductive isolation process. However, we can use divergence time estimates to gain some information about when a possible reproductive isolation has been started, even under nescience of the process that may have occurred. The divergence between the G clade and the *Bryoria fuscescens* agg. clade was 0.69 Mya, which we interpreted as caused by a reproductive isolation. Thus *Bryoria glabra* is confirmed as a species, and although sometimes can be considered morphologically cryptic, mature specimens show subtle diagnostic characters from other taxa in the section (e.g. regular branching pattern, obtuse and rounded angles between branches and regularly oval, white soralia). Instead, the relationship between the Ko, NA and WD clades was not resolved, but its split is estimated at 100 000 years, or even 60 000 if only the ITS marker is considered. Being clades that have diverged so recently, their reproductive isolation is uncertain for us. These divergence times are even more recent than the observed in the Darwin´s finches of the Galapagos Islands (Lamichhaney *et al.* 2015), an example of a very recent speciation process, and closer to the most recently diversified species with hybrid origin in the plant genus *Linaria* (Blanco–Pastor *et al.* 2012), a genus including clades with diversification rates in the range of recent plant radiation events (Fernández–Mazuecos & Vargas 2015). Other groups in *Parmeliaceae* such as *Letharia* and *Xanthoparmelia* also have relatively recent diversification histories (e.g. during the Pleistocene), and face similar challenges in accurately delimiting species boundaries (Altermann *et al.* 2014, Leavitt *et al.* 2013). It seems that when studying species with recent diversification histories, phylogenetic approach mainly fails to resolve relationships and misinterpreting these results can deceive taxonomic conclusions (Leavitt *et al.* 2016).

Despite the absence of supporting phenotypic characters, the geographical and ecological similarities, the very recent dates of divergence, and the possibility of incomplete

lineage sorting, we can consider the Ko, NA and WD clades as different, but conspecific, evolving lineages. The description of infraspecific taxa would result in fuller information of the genetic variability of a species, which may be useful in conservation procedures. The subspecies concept, although widely used in bacteriology, botany, and zoology, is now hardly used in mycology. Traditionally, the concept was used in cases of populations with geographical differences and with overlapping intermediate specimens resulting from genetic recombination (Stuessy 2009). In our case, users (e.g. ecologists and fieldworkers) could use a species name with confidence, and, if molecular data are available, a more precise identification to the subspecies level could be reached.

On the other hand, accepting and describing these phenotypically cryptic lineages at species–level formally could be the most appropriate solution. Appearance of cryptic species is a common phenomenon in the family *Parmeliaceae* and our results are in concordance with other studies in which molecular markers in combination with statistical tools revealed many genetically distinct lineages hidden under a single taxon (Alors *et al.* 2016, Del Prado *et al.* 2016, Divakar *et al.* 2016, Leavitt *et al.* 2016, Singh *et al.* 2015). Among the recognized lineages only WD clade turns out to be cosmopolitan, occurring in Europe, Asia, North America, and Africa. The other clades, NA and Ko, have a more limited distribution, having been collected so far from North America (NA), Alaska and British Columbia (Ko). Our current sampling is relatively sparse especially in South America and Africa, and does not allow conclusions about finer–scale biogeographic patterns. However, it is worth noting that the lineages NA and Ko have not been detected in our extensive sampling in Europe (Fig. 9). Practical issues (e.g. for naming older herbarium specimens or for ecological studies) could easily be resolved by recognizing the three groups as species within an aggregate, i.e. the *Bryoria fuscescens* agg. and to encourage the adoption of that suffix when precise molecular species identifications cannot be made. This is in accordance with the practice in other groups of lichens e.g. *Cladia aggregata* agg. (Parnmen *et al.* 2013), and in flowering plant groups such as the *Rubus fruticosus* agg. and the *Taraxacum officinale* agg. (Salvini *et al.* 2006, Stace 1998). We therefore adopt such a taxonomy here.

It may be significant that the Ko, NA, and WD clades origin is predicted during periods in which ice sheets, which had occupied large areas of the Northern Hemisphere, had retreated to leave the main regions in which these lichens are now distributed. Alaska could have had refugia for populations that evolved into the Ko clade (Brubaker *et al.* 2005, Anderson *et al.* 2006), whereas southern displaced specimens could have migrated northwards and evolved into the NA clade. In Europe, many refugia for plants are known in the Mediterranean peninsulas (Médail & Diadema 2009), and after ice retreated, *Bryoria* genepools evolved in

this regions could have recolonized Europe northwards, explaining the higher genetic variability found in clade WD. However, it is important to note that north–east Asia is not represented amongst our sequenced samples, but this area was once connected with both Europe and North America through the Beringian land bridge. All three clades might be co–habiting in that region, and it is possible that a continuum could appear after achieving more extensive sampling.

Our results provide strong support for rejection of the traditional characters used in this *Bryoria* complex to delimit species. However, although genetically not differentiated, consistent combinations of phenotypic characters nevertheless result in separable defined morphologies, as in pale morphs of *Bryoria capillaris* and *B. pikei*. These morphs could conceivably have been formed before the radiation of the Ko, NA and WD clades, as similar or identical *B. capillaris / B. pikei* phenotypes can be observed in all three. What produces or maintains such a characteristic morph without genetic isolation is unresolved. It should be noted that any influence of the algal symbiont can be disregarded as all material in the complex shares the same species and even same haplotypes of *Trebouxia* (Lindgren *et al.* 2014, Boluda *et al. in prep.*). Here we used neutral markers to detect gene–flow gaps between lineages, discarding genetic isolation as the reason for morphospecies existence. We recognize three hypotheses that could explain why these well characterized phenotypes are maintained within a single species.

(1) Incomplete lineage sorting. Neutral markers are useful to understand gene–flow patterns, while adaptive markers provide the evolutionary pressure that contributes to speciation. Because adaptive markers are under natural selection, they can be present in certain morphs and absent in others, even if there is gene flow amongst them (Lumley & Sperling 2011). In that case, adaptive markers could show a different evolutionary history than neutral ones, and morphospecies could emerge forming defined genetic groups. Incomplete lineage sorting processes can be common in the first steps of speciation, as seems to be the case here, supported by the inconsistent results of the loci FRBi15 and FRBi16. In *Bryoria*, supposed adaptive markers could correspond to the genes that activate the production of barbatolic and alectorialic acids or the characteristic epicortex in *B. capillaris* morphospecies (Boluda *et al*. 2014).

(2) Role of lichenicolous basidiomycete fungi. It has recently been reported that yeast morphs of the lichenicolous and gall–forming basidiomycete *Cyphobasidium* can be abundant in or on the cortex of *Bryoria* species (Spribille *et al*. 2016). The classical species distinction in that case was mainly based in the distribution of vulpinic acid through the whole thallus in *B. tortuosa*, but only in soralia and apothecia in *B. fremontii*. However, molecular data showed

them to be conspecific (Velmala *et al*. 2009). Spribille *et al*. (2016) found a possible relation between *Cyphobasidium* yeast abundance and vulpinic acid production, detecting also these yeasts in *Bryoria capillaris*. Yeasts were found living among the cortical polysaccharides of the thallus, and the variability of the surface substances, together with extrolite production, contributes to the concept of morphospecies in the *Bryoria fuscescens* agg. (Boluda *et al*. 2014). Further research is required to determine if *Cyphobasidiales* yeasts modify *Bryoria* phenotypes.

(3) Ecotypic variability. Two main morphologies can be split in the species aggregate, the mainly dark without barbatolic acid (*Bryoria fuscescens* morph) and the mainly pale with barbatolic acid (*B. capillaris* morph). They show slight differences in water holding capacity (Esseen *et al*. 2015), in protection against excess of light (Färber *et al*. 2014), and generally a different amount of epicortical substances (Boluda *et al*. 2014). This could indicate that the characteristics may be environmental adaptations, and the environment activates certain genes that result in the different morphologies. However, this does not explain why both morphs can grow together, even in physical contact and consequently, under identical environmental conditions.

In order to test which of these hypotheses are responsible of the factors that are producing different morphospecies in this group of *Bryoria*, and how geological events modeled the genetic diversity, more extensive genetic population studies of the WD clade in particular are required.

Acknowledgements

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Supplementary material

Table S1. Chemical, geographical and morphological characters used in the phenogram reconstruction (Fig. 1).

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	Alectorialic acid	Barbatolic acid	Fumarprotocetraric acid	Gyrophoric acid	Norstictic acid	Psoromic acid	Old World (0) / New World (1)	Thallus brownish (0) / whitish (1)	Branching angles acute	Branching angles obtuse	Soralia fissural	Soralia tuberculate	Pseudocyphellae inconspicuous (0) /conspicuous (1)
capillaris_L01-17	1	1	0	0	0	0	0	0	1	0	0	0	0
capillaris_L06-10	1	1	0	0	0	0	0	0	1	0	0	0	1
capillaris_L07-15	1	1	0	0	0	0	0	0	1	1	0	0	0
capillaris_L08-12	1	1	0	0	0	0	0	0	1	0	0	1	0
capillaris_L13-03	1	1	0	0	0	0	0	0	1	1	0	1	0
capillaris_L14-02	1	1	1	0	0	1	0	0	1	1	0	0	1
capillaris_L141	1	1	0	0	0	0	0	0	1	0	0	1	0
capillaris_L15-15	1	1	0	0	0	0	0	0	1	0	0	0	0
capillaris_L16-21	1	1	0	0	1	1	0	0	1	1	0	0	0
capillaris_L211	1	1	0	0	0	0	0	0	1	0	0	1	0
capillaris_L270	0	1	0	0	0	0	0	0	1	0	0	1	0
capillaris_S192	1	1	0	0	0	0	0	0	1	0	0	1	0
capillaris_S2	1	1	0	0	0	0	0	0	1	0	0	1	0
friabilis_02	0	0	0	1	0	0	1	1	1	0	0	0	1
friabilis_L355	0	0	0	1	0	0	1	1	1	0	0	0	1
friabilis_L407	0	0	0	1	0	0	1	1	1	0	0	0	1
friabilis_S395	0	0	0	1	0	0	1	1	1	0	0	0	1
fuscescens_L12-03	0	0	1	0	0	0	0	1	1	0	1	1	0
fuscescens_L12-05	0	0	1	0	0	0	0	1	0	1	1	1	0
fuscescens_L139	0	0	1	0	0	0	0	1	1	0	1	1	0
fuscescens_L149	0	0	1	0	0	0	0	1	1	0	1	1	0
fuscescens_L15-21	0	0	1	0	0	0	0	1	1	1	1	1	0
fuscescens_L160	0	0	1	0	0	0	0	1	1	0	1	1	0
fuscescens_L189	0	0	1	0	0	0	0	1	1	0	1	1	0
fuscescens_L224	0	0	1	0	0	0	0	1	1	0	1	1	0
fuscescens_L232	0	0	0	0	0	0	0	1	1	0	1	1	0
fuscescens_L305	0	0	1	0	0	0	0	1	1	0	1	1	0
fuscescens_S109	0	0	1	0	0	0	0	1	1	0	1	1	0
fuscescens_S157	0	0	1	0	0	0	0	1	1	0	1	1	0

fuscescens_S24	0	0	1	0	0	0	0	1	1	0	1	1	0
fuscescens_S256	0	0	1	0	0	0	1	1	1	0	1	1	0
fuscescens_S259	0	0	1	0	0	0	1	1	1	0	1	1	0
fuscescens_S260a	0	0	1	0	0	0	1	1	1	0	1	1	0
fuscescens_S261	0	0	1	0	0	0	1	1	1	0	1	1	0
fuscescens_S267	0	0	1	0	0	0	1	1	1	0	1	1	0
fuscescens_S272	0	0	1	0	0	0	1	1	1	0	1	1	0
fuscescens_S274	0	0	0	0	0	0	1	1	1	0	1	1	0
fuscescens_S369	0	0	1	0	0	0	1	1	1	0	1	1	0
fuscescens_S379	0	0	1	0	0	0	1	1	1	0	1	1	0
fuscescens_S380	0	0	1	0	0	0	1	1	1	0	1	1	0
fuscescens_S56	0	0	1	0	0	0	0	1	1	0	1	1	0
implexa_L01-01	0	0	0	0	0	1	0	1	1	1	1	1	1
implexa_L06-05	0	0	1	0	0	1	0	1	1	0	0	1	0
implexa_L10-03	0	0	0	0	0	1	0	1	0	1	0	0	1
implexa_L11-15	0	0	0	0	0	1	0	1	0	1	0	1	0
implexa_L16-15	0	0	0	0	0	1	0	1	1	0	0	0	1
implexa_S168	0	0	0	0	0	1	0	1	1	1	1	1	1
implexa_S22	0	0	0	0	0	1	0	1	1	1	1	1	1
implexa_S36	0	0	0	0	0	1	0	1	1	1	1	1	1
implexa_S39	0	0	0	0	0	1	0	1	1	1	1	1	1
implexa_S67	0	0	0	0	0	1	0	1	1	1	1	1	1
inactiva_L206	0	0	0	0	0	0	1	0	1	0	0	0	1
inactiva_L323b	0	0	0	0	0	0	1	0	1	0	0	0	1
inactiva_L347	0	0	0	0	0	0	1	0	1	0	0	0	1
inactiva_L358	0	0	0	0	0	0	1	0	1	0	0	0	1
inactiva_S239a	0	0	0	0	0	0	1	0	1	0	0	0	1
inactiva_S384	0	0	0	0	0	0	1	0	1	0	0	0	1
inactiva_S392	0	0	0	0	0	0	1	0	1	0	0	0	1
kockiana_L394	0	0	0	0	0	1	1	0	1	1	0	0	0
kockiana_L396	0	0	0	0	0	1	1	0	1	1	0	0	0
kuemmerleana_L04-03	0	0	0	0	1	0	0	1	1	0	0	0	0
kuemmerleana_L09-04	0	0	0	0	1	0	0	1	1	1	0	0	1
kuemmerleana_L09-07	0	0	0	0	1	0	0	1	1	0	0	0	0
kuemmerleana_L16-17	0	0	0	0	1	1	0	1	1	1	1	1	1
kuemmerleana_L244	0	0	0	0	1	0	0	1	1	1	1	1	1
kuemmerleana_L274	0	0	0	0	1	0	0	1	1	1	1	1	1
kuemmerleana_L275	0	0	0	0	1	0	0	1	1	1	1	1	1
kuemmerleana_S128	0	0	0	0	1	0	0	1	1	1	1	1	1
kuemmerleana_S160	0	0	0	0	1	0	0	1	1	1	1	1	1
pikei_02	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_04	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_05	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_07	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_09	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_10	1	1	0	0	0	0	1	1	1	0	0	0	1

pikei_11	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_12	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_13	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_14	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_15	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_a	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_b	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_c	1	0	0	0	0	0	1	1	1	0	0	0	1
pikei_d	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_L197	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_L210	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_L241	1	0	0	0	0	0	1	1	1	0	0	0	1
pikei_L374	1	0	0	1	0	0	1	1	1	0	0	0	1
pikei_L376	1	0	0	1	0	0	1	1	1	0	0	0	1
pikei_L377	1	0	0	1	0	0	1	1	1	0	0	0	1
pikei_S221	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_S362	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_S368	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_S382	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_S383a	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_S390	1	1	0	0	0	0	1	1	1	0	0	0	1
pikei_S394	1	1	0	0	0	0	1	1	1	0	0	0	1
pseudofuscescens_S222	0	0	0	0	1	0	1	0	1	1	0	0	1
pseudofuscescens_S232	0	0	0	0	1	0	1	0	1	1	0	0	1
pseudofuscescens_S370	0	0	0	0	1	0	1	0	1	1	0	0	1
pseudofuscescens_S371	0	0	0	0	1	0	1	0	1	1	0	0	1
pseudofuscescens_S377	0	0	0	0	1	0	1	0	1	1	0	0	1
pseudofuscescens_S386	0	0	0	0	1	0	1	0	1	1	0	0	1
pseudofuscescens_S387	0	0	0	0	1	0	1	0	1	1	0	0	1
sp_L395	0	0	0	0	0	0	1	0	1	1	0	0	0
sp_S392	0	0	0	0	0	0	1	0	1	1	0	0	0
vrangiana_L02-20	0	0	1	0	0	0	0	1	0	1	1	1	0
vrangiana_L03-07	0	0	1	0	0	0	0	1	0	1	0	1	0
vrangiana_L05-17	0	0	1	0	0	0	0	1	0	1	0	1	0
vrangiana_L07-03	0	0	1	0	0	0	0	1	1	1	1	1	1
vrangiana_L07-19	0	0	0	0	0	0	0	1	0	1	0	0	0
vrangiana_L08-19	0	0	0	0	0	0	0	1	0	1	1	1	0
vrangiana_L08-20	0	0	1	0	0	0	0	1	0	1	0	1	0
vrangiana_L10-13	0	0	0	0	0	0	0	1	0	1	0	0	0
vrangiana_L12-11	0	0	1	1	0	0	0	1	0	1	1	1	0
vrangiana_L13-12	0	0	0	1	0	0	0	1	1	1	0	0	1
vrangiana_L272	0	0	0	1	0	0	0	1	0	1	1	1	0
vrangiana_L273	0	0	0	0	0	0	0	1	0	1	1	1	0
vrangiana_L300	0	0	0	0	0	0	0	1	0	1	1	1	0
vrangiana_L307	0	0	0	1	0	0	0	1	0	1	1	1	0
vrangiana_S10	0	0	0	1	0	0	0	1	0	1	1	1	0

vrangiana_S164	0	0	0	0	0	0	0	1	0	1	1	1	0
vrangiana_S166	0	0	1	0	0	0	0	1	0	1	1	1	0
vrangiana_S196a	0	0	0	0	0	0	0	1	0	1	1	1	0
vrangiana_S341	0	0	0	0	0	0	0	1	0	1	1	1	0
vrangiana_S385	0	0	1	0	0	0	0	1	0	1	1	1	0
vrangiana_S42	0	0	0	1	0	0	0	1	0	1	1	1	0
vrangiana_S45	0	0	0	0	0	0	0	1	0	1	1	1	0
vrangiana_S57	0	0	1	0	0	0	0	1	0	1	1	1	0
vrangiana_S59	0	0	1	0	0	0	0	1	0	1	1	1	0
vrangiana_S6	0	0	1	0	0	0	0	1	0	1	1	1	0
vrangiana_S62	0	0	0	1	0	0	0	1	0	1	1	1	0
vrangiana_S72	0	0	0	0	0	0	0	1	0	1	1	1	0

Table S2. Microsatellite fragment lengths of analyzed specimens.

Sample	Bi1	Bi03	Bi04	Bi05	Bi10	Bi11	Bi12	Bi14	Bi19
capillaris_L01-17	103	279	323	128	437	316	100	365	346
capillaris_L06-10	103	279	323	128	437	316	100	365	346
capillaris_L07-15	103	279	323	128	437	316	100	365	346
capillaris_L08-12	103	279	323	128	434	316	103	361	346
capillaris_L13-03	94	279	325	138	434	318	115	361	346
capillaris_L14-02	112	279	327	128	437	320	100	365	346
capillaris_L141	123	281	323	136	434	316	100	361	346
capillaris_L15-15	112	279	323	-	437	316	100	365	346
capillaris_L16-21	103	279	323	128	437	316	100	365	346
capillaris_L211	112	279	323	136	434	316	103	361	346
capillaris_L270	112	279	323	138	434	316	124	361	346
capillaris_S192	112	279	323	128	437	316	100	365	346
capillaris_S2	94	279	323	138	434	316	124	361	346
friabilis_02	114	281	317	137	434	310	100	-	350
friabilis_L355	120	277	316	137	436	310	100	365	350
friabilis_L407	132	281	316	137	434	310	131	365	346
friabilis_S395	109	277	316	132	438	310	100	365	350
fuscescens_L12-03	112	279	323	138	434	316	103	361	352
fuscescens_L12-05	94	281	323	138	434	316	103	361	350
fuscescens_L139	94	279	323	138	434	316	103	361	352
fuscescens_L149	123	279	323	138	434	316	103	361	350
fuscescens_L15-21	123	277	325	138	434	318	115	361	352
fuscescens_L160	94	281	323	136	434	316	103	361	352
fuscescens_L189	112	279	323	138	434	316	100	361	352
fuscescens_L224	112	279	323	138	434	316	103	361	352
fuscescens_L232	94	279	323	138	434	316	103	361	352
fuscescens_L305	117	279	323	138	434	316	137	361	352
fuscescens_S109	112	281	323	138	434	316	118	361	350
fuscescens_S157	117	279	323	136	434	316	103	361	350
fuscescens_S24	112	281	323	138	434	316	106	361	352
fuscescens_S256	94	281	323	-	434	316	124	363	354
fuscescens_S259	94	277	323	138	437	316	103	363	352
fuscescens_S260a	82	279	323	138	437	316	103	363	352
fuscescens_S261	82	279	323	138	437	316	103	363	352
fuscescens_S267	82	279	323	138	437	316	103	363	352
fuscescens_S272	82	279	323	138	437	316	103	363	352
fuscescens_S274	94	279	323	138	434	316	-	361	350
fuscescens_S369	-	279	323	138	437	316	103	363	352
fuscescens_S379	94	279	323	138	437	316	100	363	352
fuscescens_S380	112	279	323	138	434	316	118	361	352
fuscescens_S56	123	279	323	136	434	316	100	361	350
glabra_01	114	283	306	132	426	299	115	369	344
glabra_02	120	283	306	132	426	299	-	369	344
glabra_03	120	283	306	132	426	299	-	369	344
glabra_04	120	283	306	132	426	299	-	369	344
glabra_05	120	283	306	132	426	299	-	369	344

glabra_L186	114	283	304	132	426	297	115	371	344
glabra_L406	114	283	304	132	426	297	115	371	344
glabra_L414	114	283	306	132	426	299	115	371	344
glabra_S388	114	283	306	132	426	299	115	371	344
implexa_L01-01	135	281	323	138	434	316	106	361	350
implexa_L06-05	112	263	323	136	434	316	103	361	350
implexa_L10-03	106	279	323	128	437	316	100	365	346
implexa_L11-15	94	279	323	136	435	316	103	361	352
implexa_L16-15	112	281	325	138	434	318	115	361	352
implexa_S168	112	279	323	138	434	316	115	361	350
implexa_S22	94	279	323	136	436	316	118	361	352
implexa_S36	94	279	325	136	434	318	103	361	346
implexa_S39	-	-	323	138	434	316	-	361	350
implexa_S67	94	279	325	136	434	318	103	361	346
inactiva_L206	114	277	317	137	434	311	100	365	350
inactiva_L323b	114	281	316	132	434	310	131	365	350
inactiva_L347	114	277	317	132	434	310	124	365	350
inactiva_L358	109	281	317	132	436	310	106	365	350
inactiva_S239a	109	277	314	132	434	308	100	365	350
inactiva_S384	114	277	316	137	434	310	100	365	350
inactiva_S392	120	281	316	137	434	310	100	365	350
kockiana_L394	94	279	317	136	472	310	109	365	344
kockiana_L396	94	279	317	136	472	310	109	365	344
kuemmerleana_L04-03	129	279	325	138	434	318	103	361	352
kuemmerleana_L09-04	112	281	323	128	437	316	100	365	346
kuemmerleana_L09-07	112	281	323	128	437	316	100	365	346
kuemmerleana_L16-17	112	281	323	138	434	316	106	361	352
kuemmerleana_L244	117	279	323	138	434	316	115	361	350
kuemmerleana_L274	94	279	323	136	434	316	103	361	352
kuemmerleana_L275	94	279	323	136	434	316	103	361	352
kuemmerleana_S128	100	279	323	136	434	316	100	361	354
kuemmerleana_S160	117	279	323	138	434	316	103	361	350
pikei_02	117	277	317	137	434	310	100	365	344
pikei_04	117	277	317	137	434	310	100	365	344
pikei_05	117	277	317	137	434	310	100	365	344
pikei_07	117	281	317	137	434	311	109	-	344
pikei_09	114	281	317	132	-	310	100	-	344
pikei_10	117	277	317	137	434	311	100	365	344
pikei_11	114	277	317	137	434	310	100	365	344
pikei_12	114	277	-	137	434	-	100	-	344
pikei_13	-	277	317	137	434	310	118	365	344
pikei_14	117	277	317	137	434	310	127	365	344
pikei_15	114	281	317	137	436	310	109	-	344
pikei_a	114	-	323	132	437	316	100	-	346
pikei_b	117	277	317	137	434	311	100	365	344
pikei_c	108	-	-	137	-	314	100	326	344
pikei_d	117	277	-	137	437	310	100	-	344
pikei_L197	114	277	316	132	434	310	109	365	344
pikei_L210	109	277	316	132	434	310	100	365	344

pikei_L241	126	277	316	137	434	310	109	-	352
pikei_L374	114	277	316	137	434	310	106	365	344
pikei_L376	132	277	316	137	434	310	127	365	352
pikei_L377	109	277	316	137	434	310	103	365	344
pikei_S221	109	277	316	132	436	310	106	365	352
pikei_S362	114	281	317	137	434	311	100	365	344
pikei_S368	112	281	316	132	436	310	109	365	344
pikei_S382	114	281	317	137	434	311	100	365	344
pikei_S383a	114	281	317	132	436	311	100	365	344
pikei_S390	114	277	316	137	434	310	100	365	344
pikei_S394	126	277	316	137	434	310	100	365	352
pseudofuscescens_S222	-	277	316	132	-	310	100	365	-
pseudofuscescens_S232	114	277	317	137	436	310	100	365	350
pseudofuscescens_S370	117	277	316	132	434	310	100	365	350
pseudofuscescens_S371	117	281	316	132	434	310	100	365	350
pseudofuscescens_S377	-	277	-	132	434	311	100	365	-
pseudofuscescens_S386	112	277	317	132	438	311	100	365	350
pseudofuscescens_S387	117	277	316	132	434	310	127	365	350
sp_L395	94	273	317	136	460	310	109	365	344
sp_S392	97	283	317	136	472	310	118	365	344
vrangiana_L02-20	112	279	323	138	434	316	106	361	352
vrangiana_L03-07	94	279	325	136	434	318	115	365	346
vrangiana_L05-17	117	281	323	138	434	316	121	361	350
vrangiana_L07-03	94	281	323	138	434	316	118	361	352
vrangiana_L07-19	123	279	323	138	434	316	103	361	350
vrangiana_L08-19	94	281	323	136	436	316	103	361	352
vrangiana_L08-20	94	279	323	138	434	316	121	361	352
vrangiana_L10-13	123	283	323	128	437	316	100	365	346
vrangiana_L12-11	94	279	323	138	434	316	144	361	352
vrangiana_L13-12	94	279	323	138	434	316	103	361	352
vrangiana_L272	112	279	323	136	434	316	-	361	350
vrangiana_L273	94	279	323	138	436	316	103	361	350
vrangiana_L300	94	281	323	136	434	316	115	361	350
vrangiana_L307	123	279	325	138	434	318	103	361	352
vrangiana_S10	123	279	323	136	434	316	124	361	352
vrangiana_S164	94	279	323	136	434	316	127	361	350
vrangiana_S166	117	279	323	138	434	316	144	365	352
vrangiana_S196a	94	281	323	138	434	316	118	361	350
vrangiana_S341	-	279	323	-	434	316	-	361	350
vrangiana_S385	94	279	323	138	434	316	103	361	350
vrangiana_S42	117	279	323	138	434	316	131	361	352
vrangiana_S45	112	279	323	136	434	316	100	361	350
vrangiana_S57	94	279	323	138	434	316	115	361	352
vrangiana_S59	94	279	323	138	434	316	115	361	352
vrangiana_S6	123	279	323	138	434	316	103	361	350
vrangiana_S62	112	279	323	138	434	316	115	361	352
vrangiana_S72	94	279	323	136	436	316	103	361	352

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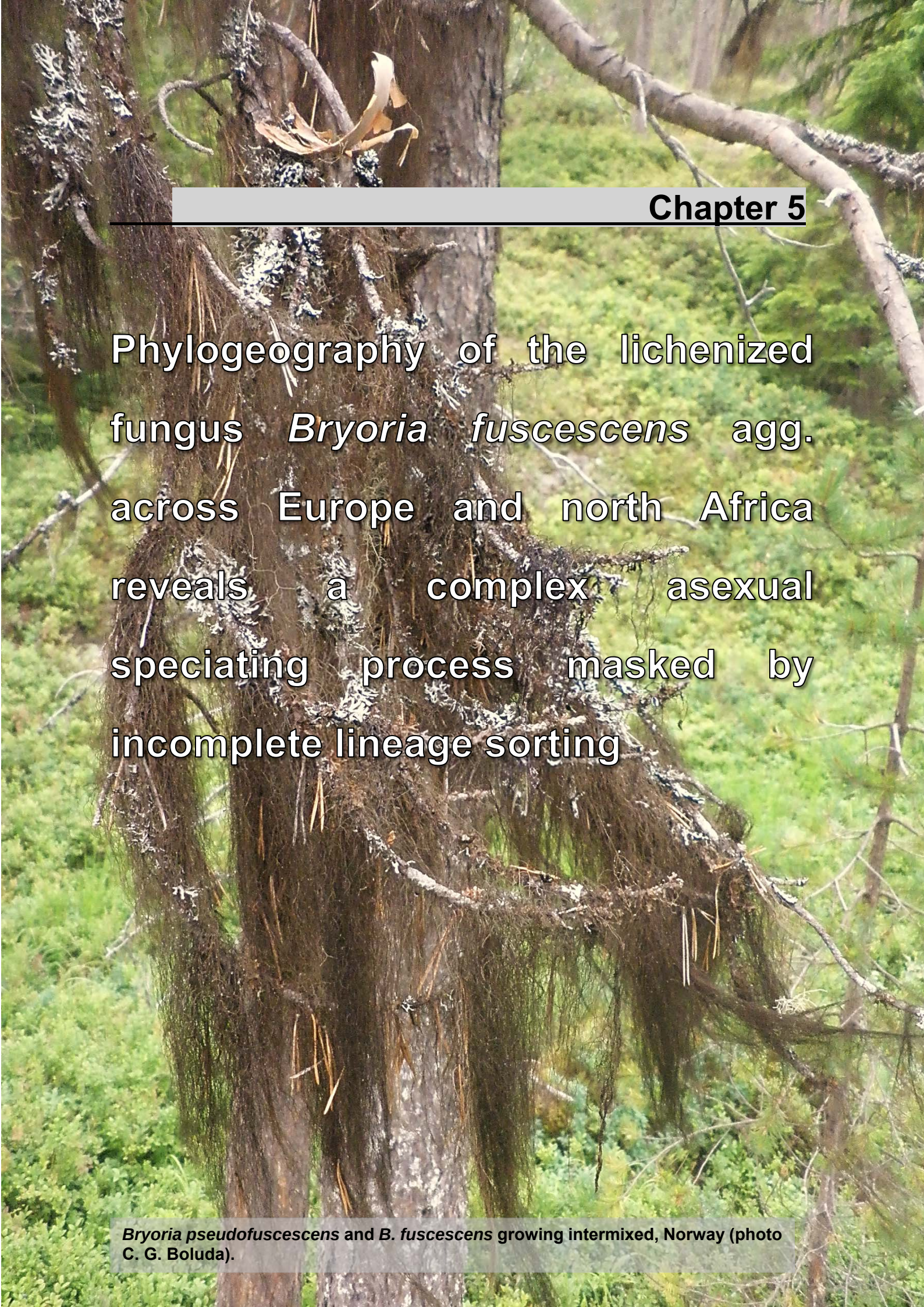

Chapter 5

Phylogeography of the lichenized fungus *Bryoria fuscescens* agg. across Europe and north Africa reveals a complex asexual speciating process masked by incomplete lineage sorting

Bryoria pseudofuscescens **and** ***B. fuscescens*** **growing intermixed, Norway (photo C. G. Boluda).**

Introduction

During the last 2.5 My, Europe has been strongly affected by glacial and interglacial periods (Zachos *et al.* 2001). Through the strongest glaciations, ice sheets covered northern and great part of central Europe, leaving huge areas available for species recolonization after ice melt in the interglacial epochs (Tzedakis *et al.* 2013). The extreme and fast environmental fluctuations influenced the phylogeographical patterns of native taxa. Accordingly, different authors have described three recurrent main groups of refuge areas for plants and animals for the coolest periods: Iberian, Italian and Balkan peninsulas (Bennett *et al*. 1991; Petit *et al.* 2003; Feliner 2011). Frome these refuges, the populations could have recolonized northern areas in the interglacial periods (Taberlet *et al.* 1998; Godfrey 1999). The most comprehensive phylogeographical works performed in lichens along Europe (using the species *Lobaria pulmonaria*; Widmer *et al*. 2012), show similar patterns as in plants and animals. However, we cannot overlook the potential significance of the described cryptic refugia on the coasts of northern Europe, not only for polar taxa, but also for forest ecosystems (Willis & van Andel 2004; Birks & Willis 2008).

The strong climate changes could produce the extinction of many species, but also favoured allopatric speciation in others. Allopatry may be caused by both, glacial and interglacial periods. In glacial periods one species may split into geographically isolated refugee areas, however in the interglacial period a boreal species may migrate to north leaving southern specimens isolated in the higher mountains, (Linares 2011; Roberts & Hamann 2015). Climatic variation occurred so fast that populations that were usually isolated did not had time for speciation. However, for fast evolving groups, as insects or annual herbs, the geographical isolation may conduct to the emergence of new taxa (Weir & Schluter 2004; Veith *et al*. 2003).

For asexual and morphologically variable species, as in our species, putting aside the natural selection, we can expect genetic drift as one of the main evolutionary forces. The population size is a very important factor for speciation in that kind of taxa. In small populations, newly originated phenotypes can be fixed merely by genetic drift (Masel 2011). In large populations, new phenotypes or genotypes could be maintained mixed with others, producing variable species, but the probability to fix one of these phenotypes in all the specimens (a speciation event) is extremely low if it is not favoured by natural selection. Small populations or founder effects are important processes in species under mutational drift, and these events are typically produced during glacial and interglacial periods. Events as population size

variation, isolation, interbreeding, bottlenecks, colonisation events, or areas with glacial refugia, leave signals in the genomes of a species. These allow us to draw a history of the species, and in the cases where speciation occurred during these events, its history could tell us the causes of speciation. Whereas a speciating event in a phylogeographical framework has been studied for some plants and animals (Moritz *et al.* 1992; Avise & Walker 1998; Avise *et al.* 1998), it has never been deeply analysed in lichens.

Lichenized fungi of the *Bryoria fuscescens* agg. are closely linked to boreal forests, one of the most affected ecosystems by recent glacial events and human activities. This group contains three recently evolved asexual and cryptic species (*Bryoria fuscescens*, *B. pseudofuscescens*, and *B. kockiana*), with an estimated origin around 100 000 years ago, at the end of the last interglacial period (Chapter 4). These species are endemic to north America, except *Bryoria fuscescens*, which has also been found in Europe, Asia and Africa. *Bryoria fuscescens* is relatively a common species in the forests of north Europe, especially in Scandinavia, but is restricted to high and humid mountains in central and especially southern Europe, where it is rare. *Bryoria fuscescens* is a morphological and chemical variable species, including synonyms of up to 11 taxa described in the past centuries only in Europe. However, specimens can be usually gathered into two main phenotypes: (i) the *fuscescens* phenotype, mainly from pale brown to black, frequently sorediate, and never containing barbatolic and alectorialic acids, and (ii) the *capillaris* phenotype, mainly pale coloured, rarely sorediate, and always producing barbatolic and alectorialic acids. Eariler, these two morphologies were considered as distinct species, however, in a recent study population of these taxa were found genetically intermixed (*cf.* Chapter 4). Both morphs can be found growing in physical contact on the same tree branch, indicating that environmental factors are not responsible for producing the phenotypes. Whereas previous studies showed that DNA sequences were more related with continental isolation than phenotypic differences, the microsatellites revealed a small trend to split *fuscescens* and *capillaris* morphs into weakly isolated genepools (Chapter 4). Then, evolutive patterns not shown by DNA sequences or in studies performed with a relative low sampling could remain hidden.

Bryoria fuscescens s. str. has a special evolutive framework to test a putative speciation process towards *capillaris* and *fuscescens* phenotypes in a phylogeographical scenario. Moreover, this species is frequently used as indicator of forest quality (Esseen *et al.* 1996), in regression on southern and Central Europe, and expected to be strongly affected by global warming. The knowledge of the past and present population dynamics could help us to study a putative lichen speciating process, and predict how the global warming may affect to this species and lichens in general growing in similar habitats. Furthermore, the way in what lichens are affected by habitat fragmentation, human interactions and its own dispersal capacities are

insufficiently understood, which is necessary to improve the protection measurements of this kind of flora. To study the evolution, phylogeography and population dynamics of *Bryoria fuscescens* agg., 1400 morphologically variable samples were collected along the Euro-Mediterranean region, and analyzed with eigth DNA sequences loci and 18 fungal-specific microsatellite markers (Nadyeina *et al.* 2015).

Materials and Methods

Sampling and specimen characteristics

A total of 1400 *Bryoria fuscescens* thalli were collected from 64 populations across Europe, the Mediterranean Region, and the Canary Islands (Fig. 1 & Table S1). Populations were considered as distant areas of a few hundred square meters of trees or rocks containing specimens. With some exceptions, individual populations were more than 50 km apart. Around 20 specimens per population were collected (Table S1). The 64 populations were grouped to subordinate geographical regions: Scandinavia, Great Britain, Carpathians, Alps, Iberia, Mediterranean (Western Mediterranean), and Africa (Fig. 1). Within saxicolous populations, individual specimens were collected as far apart as possible, to avoid collecting putative clonal thalli (e.g. specimens located lower or close to others). In forests, different morphologies were collected from twigs, branches and trunks when present, and at different orientations and heights in order to minimize possibilities of clonality and maximize habitat diversity. For each population, pictures were taken to show factors that might affect genetic structure (e.g. specimen abundance and distribution, competition for the substrata, human activities, etc). Samples were air dried and frozen (down to –20ºC) until the analysis. For each specimen, the following characters were recorded: chemotype, soralia type (absent, tuberculate, or fissural), and presence of pseudocyphellae, apothecia and the lichenicolous fungus *Raesaenenia huuskonenii* (at × 65). Chemical products were assayed by thin layer chromatography (TLC) following Orange *et al*. (2010) and using solvent system C (200 ml toluene / 30 ml acetic acid), with concentrated acetone extracts eluted at 50 ºC and spotted onto silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany). Spotted sheets were dried for 10 min in an acetic acid atmosphere to maximize resolution. The same lichen fragment used for TLC was also used for DNA extraction to eliminate the risk of taking samples from mixed collections.

DNA extraction and PCR conditions

A single terminal branch per specimen (the same used for TLC analysis) was used for DNA extraction with the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). Each specimen was genotyped with eighteen fungus-specific microsatellite markers (Bi01-Bi16, Bi018 and Bi019) following Nadyeina *et al.* 2014. Fragment lengths were determined on an ABI PRISM® 3130 Genetic Analyser (Life Technologies, Foster City, CA, USA) using fluorescently labelled primers. Electropherograms were analyzed with LIZ-500 as internal size standard and GeneMapper 3.7 (Applied Biosystems, Foster City, CA, USA).

To confirm the sample identification and better understand the genetic relations among phenotypically distant samples, a phylogenetic analysis of 35 specimens representing the disparate phenotypes and geographical regions was done (Table S2). Eight fungal markers were used, three of them commonly employed in barcoding (ITS, IGS, and GAPDH), and five newly designed for this study (FRBi13, FRBi15, FRBi16, FRBi18, and FRBi19; Table S3). These new markers specific for *Bryoria* section *Implexae* were obtained from the flanking regions of the microsatellites, which were variable non–coding DNA fragments that can contain phylogenetic signal (Devkota *et al.* 2014). The flanking regions of the 18 microsatellite markers were checked upstream and downstream in the 454 pyrosequencing contigs used to obtain the microsatellites in Nadyeina *et al.* (2014). The variability of each region was assessed with the number of variable sites in contigs supported by 2 to 16 copies. Of the 36 regions (two for each one of the 18 microsatellites), the five most variable were those of the microsatellites Bi13, Bi15, Bi16, Bi18 and Bi19, and these were selected as phylogenetic markers (Table S3). Primers were designed with Primer 3 Plus (Untergasser *et al.* 2007). Markers of the microsatellites Bi15 and Bi16 were previously used in Boluda *et al.* (2017). Additionally, to detect any possible algal partner species variability among the genepools or phenotypes, the *Trebouxia* ITS loci of the 35 selected specimens were sequenced with the primers ITS1T and ITS4T (Kroken & Taylor 2000).

A reaction mixture of 25 µl, containing 12 µl sterile water, 9 µl JumpStartTM REDTaq® ReadyMixTM PCR Reaction Mix (Sigma–Aldrich, St. Louis, MI, USA), 1.25 µl of each primer forward and reverse at 10 µM, and 1.5 µl DNA template, were used for PCR. Cycling conditions for ITS, GAPDH, FRBi13, FRBi15, FRBi16 and FRBi19 were 2 min at 94 ºC; 35 cycles of 30 s at 94 ºC, 30 s at 56 ºC; 2 min at 72 ºC; and a final extension of 5 min at 72 ºC. For FRBi18 and algal ITS cycling conditions were the same as above but with an annealing temperature of 50 ºC. For IGS, the cycling process used was: 2 min at 94 ºC; then 15 cycles of 30 s at 94 ºC, 30 s at 55 ºC (decreasing 1 ºC each cycle down to 40 °C); 2 min at 72 ºC, then 35 cycles of 30 s at 94 ºC, 30 s at 55 ºC; 90 s at 72 ºC; and a final extension of 5 min at 72 ºC. PCR products

were checked and quantified on 1 % agarose gel stained with ethidium bromide, and cleaned using Exonuclease I and FastAPTM Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer´s instructions. Sequencing was performed with labeling using BigDye Terminator v.3.1 Kit (Applied Biosystems), as follows: 25 cycles of 20 s at 96 ºC, 5 s at 50 ºC, and 2 min at 60 ºC. Clean–up used BigDye XTerminator Purification Kit (Applied Biosystems), according to the manufacturer´s instructions. Sequences were obtained in an ABI PRISM® 3130 Genetic Analyser (Life Technologies) and manually adjusted using DNA Workbench (CLC bio, Aarhus, Denmark) and MEGA5 (Tamura *et al.* 2011).

Genetic diversity and population dynamics

For each population and population group, allelic richness (AR) and private allelic richness (PAR) parameters were estimated using a rarefaction approach with ADZE (Szpiech *et al*. 2008). Nei's unbiased haploid diversity (uh) was calculated using GenAlEx v.6.41 (Peakall & Smouse 2006). Levels of linkage disequilibrium were estimated using the index rBarD (unbiased measure of linkage disequilibrium), which corresponds to the index of association (IA) but is independent of the number of loci included (Agapow & Burt 2001). rBarD was calculated within populations and among loci with the R package *poppr* v.2.1.1 (Kamvar *et al.* 2014, 2015), which is appropriate for haploid species with both clonal and sexual reproductive modes. The null model of no linkage between loci was performed with 999 permutations and with a significance level of 0.05. This is expected to be 0 if populations are freely recombining (sexual reproduction) and significantly greater than 0 if the alleles are associated (asexual reproduction). To check for any possible population expansion or diminution, Microsoft Excel macro KGTESTS (Bilgin 2007) was used at population and regional levels. The analysis compared observed and expected microsatellite allelic distributions under a mutation-drift equilibrium. A significant negative value in the k-test indicates expansion and a significant positive value indicate stagnation. The genetic differentiation among and within populations was tested with an analysis of molecular variance (AMOVA) as weighted average over loci with the program Arlequin v.3.5 (Excoffier & Lischer 2010). Significance test was obtained using a non-parametric permutation process with 20 022 permutations.

Genetic structure searching

To detect genepools among the specimens analyzed, two approaches were tested: (i) DAPC (Discriminant Analysis of Principal Components; Jombart *et al*. 2010), and (ii) STRUCTURE v.2.3.4 (Pritchard *et al*. 2000; Falush *et al*. 2003). DAPC, unlike STRUCTURE, does not assume unlinked markers and panmictic populations, which is highly expected in

lichens. DAPC analysis was performed using the R package *adegenet* 2.0.1 (Jombart 2008; Jombart *et al*. 2010; Jombart & Ahmed 2011), with the microsatellite data (clean data in Table 1; Table S4). STRUCTURE was run with the same data performing 100 000 burn-in generations, 100 000 iterations, and using a *k* value from 1 to 10 with 20 replicates for each *k* value. To combine the 20 runs of each *k* in a single result, CLUMMP v.1.1.2 (Jakobsson & Rosenberg 2007) was used, and visualized replacing the CLUMMP output values in a STRUCTURE output of the same *k*, plotted using the STRUCTURE software. To show the probability of each *k* value, STRUCTURE HARVESTER (Earl & von Holdt 2012), with the ΔK method (Evanno *et al.* 2005) was used, considering the most probable *k* the first one that appears close to 0 in the output graphic.

Spatial analyses

To check if geographical distance acted as a dispersal barrier, an isolation by distance (IBD) test was carried out using the R package *adegenet* (Jombart 2008; Jombart *et al*. 2010; Jombart & Ahmed 2011). Genetic Edward´s distances and geographic Euclidean distances were tested for correlation with a Mantel test using the *mantel.randtest* function. Results were plotted with the R package MASS function *kde2d* (Venables & Ripley 2002) for a better visualization of the local density points.

To examine possible asymmetric patterns of gene flow between pairs of geographical regions, MIGRATE-N 3.2.6 (Beerli & Palczewski 2010) was used. The mutation scaled immigration rate M (M = *m*/μ, *m* = immigration rate, μ = mutation rate) was estimated assuming an unknown μ, set as identical in all populations. An unconstrained migration model was used to estimate M and Θ (genetic diversity, $\Theta = 2N_e \mu$, N_e = effective population size) for each pair of populations separately, using a uniform prior for both (M = 0 – 1000, Θ = 0, 100) divided into 1 500 bins. Four Metropolis-coupled Monte-Carlo chains were set with four temperatures (1, 1.2, 3, and 1 000 000) and run for 2 million generations recording every 200th steep (10 000 steps for each run) after a burn-in period of 2500 generations. Asymmetric rates of possible immigrations between the regions were compared using M (the mode of the posterior distribution across all loci) and the number of immigrants per generation (2Nm), which is the product of M and Θ of the recipient population (Moeller *et al.* 2011).

Potential distribution estimation

To test if specimens from distinct genepools or chemotypes show different climatic correlations, and test putative changes in the past distribution of *Bryoria fuscescens*, a mapping of the potential distribution was estimated using Maxent v.3.3.3a (Philips *et al.* 2006).

Grids of the current global climatic conditions (spatial resolution of 30 arcsec, about 1 Km^2), Mid Holocene (6 000 years ago, spatial resolution of 2.5 minutes), Last Glacial Maximum (22 000 ya, 2.5 minutes) and Last Interglacial (approx. 130 000 ya, 30 arcsec) were downloaded from the WordClim database (Hijmans *et al.* 2005). The next 19 layers of bioclimatic variables were obtained: BIO1 = Annual Mean Temperature, BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp)), BIO3 = Isothermality (BIO2/BIO7) (* 100), BIO4 = Temperature Seasonality (standard deviation *100), BIO5 = Max Temperature of Warmest Month, BIO6 = Min Temperature of Coldest Month, BIO7 = Temperature Annual Range (BIO5-BIO6), BIO8 = Mean Temperature of Wettest Quarter, BIO9 = Mean Temperature of Driest Quarter, BIO10 = Mean Temperature of Warmest Quarter, BIO11 = Mean Temperature of Coldest Quarter, BIO12 = Annual Precipitation, BIO13 = Precipitation of Wettest Month, BIO14 = Precipitation of Driest Month, BIO15 = Precipitation Seasonality (Coefficient of Variation), BIO16 = Precipitation of Wettest Quarter, BIO17 = Precipitation of Driest Quarter, BIO18 = Precipitation of Warmest Quarter, BIO19 = Precipitation of Coldest Quarter. Maps in BIL format were transformed to Esri.asc format using DIVA-GIS (Hijmans *et al.* 2004), whereas tiff maps were converted into Esri.asc using the R packages tiff and raster (R Core Team 2013; Hijmans & Jacob 2012). Maps for each variable were merged to obtain a single layer per variable. To select the most relevant set of environmental variables and discard auto-correlated ones, the R package MaxentVariableSelection was used. Two Maxent analyses per genepool were run, one with all the variables and another with the most relevant. Each analysis was run 10 times, representing the median value of all runs. For past potential distribution analyses, ArcGIS® was used to build an input text with “samples with data” SWD format.

Phylogenetic reconstruction

Alignments for each locus were performed using MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/; Katoh & Standley 2013) with the G–INS–i alignment algorithm, a ‘1PAM/K = 2’ scoring matrix, with an offset value of 0.1, and the remaining parameters set as default. Alignments were deposited in TreeBASE under accession nos. TB2:S20593 (ITS, IGS and GAPDH), TB2:S20588 (FRBi13), TB2:S20589 (FRBi15), TB2:S20590 (FRBi16), TB2:S20591 (FRBi18), and TB2:S20592 (FRBi19). Partitionfinder (Lanfear *et al.* 2012) was used to detect possible intra–locus evolutionary model variability, resulting in the splitting of the fungal ITS region into ITS1, 5.8S, and ITS2, and coding each codon position separately in GAPDH. Models of DNA sequence evolution for each locus partition were selected with the program jModeltest 2.0 (Darriba *et al.* 2012), using the Akaike information criterion (AIC; Akaike 1974). The best–fit model of evolution obtained was: ITS1 = TIM2, 5.8S = K80, ITS2 = TIM2ef + G, IGS = TrN + I, GAPDH 1st position = TrN + I, GAPDH 2nd position = F81 + I, GAPDH 3th position = TPM3uf, FRBi13 = TrN, FRBi15 = TPM3uf + I,

FRBi16 = TPM3uf + G, FRBi18 = HKY, FRBi19 = TPM3uf + I. To detect any possible topological conflicts amongst loci, the CADM test (Legendre & Lapointe 2004; Campbell *et al.* 2011) was performed using the function 'CADM.global' implemented in the library ‘ape’ of R (Paradis *et al.* 2004). The five FRBi loci were not congruent among them and phylogenetic reconstructions were estimated for each one separately. Loci ITS, IGS and GAPDH were concatenated. For the concatenated matrix, specimens with more than one locus missing were excluded. Datasets were analyzed using maximum likelihood and Bayesian (B/MCMCMC) approaches with gaps treated as missing.

Bayesian reconstruction was performed with MrBayes v.3.2.1 (Ronquist & Huelsenbeck 2003). Two simultaneous runs with ten million generations each, starting with a random tree and employing twelve simultaneous chains, were executed. Every 500th tree was saved to a file. To avoid an overestimation of branch lengths the uniform compound Dirichlet prior brlenspr=unconstrained:gammadir (1, 1, 1, 1) was used (Zamora *et al.* 2015). The log–likelihood scores of sample points against generations were plotted using Tracer v.1.5 (Rambaut *et al.* 2014) and determined that stationarity had been achieved when the log–likelihood values of the sample points reached an equilibrium value and ESS values exceeded 200 (Huelsenbeck & Ronquist 2001). Posterior probabilities (PPs) were obtained from the 50 % majority rule consensus of sampled trees after excluding the initial 25 % as burn–in. The phylogenetic tree was drawn with FigTree v.1.4 (Rambaut 2009).

Results

Genetic diversity

SSRs amplifications (Table 1) showed allele sizes not expected by changes in the number of repetitions. To avoid incorporating mutations from the microsatellite flanking regions, which could have a different evolutionary pattern than the microsatellites, unexpected sizes were removed. Specimens with missing data were also removed. From the 18 loci and 1400 specimens, the final dataset without missing data included 14 loci and 1359 specimens (Table 1; Table S4).

Table 1. Amplified and analysed SSRs. Left: Number of specimens with successful amplification for each locus, and its respective number of alleles. Right: Selected loci and specimens for the analyses after remove unexpected alleles and specimens with missing data.

	Amplified SSRs		SSRs used for the analyses	
Locus	**Specimens**	**Alleles**	**Specimens**	**Alleles**
Bi01	1384	22	Not used	Not used
Bi02	1123	6	Not used	Not used
Bi03	1391	5	1359	5
Bi04	1388	8	1359	7
Bi05	1359	14	1359	10
Bi06	1366	22	1359	21
Bi07	1368	6	1359	6
Bi08	1385	5	1359	5
Bi09	597	3	Not used	Not used
Bi10	1393	5	1359	3
Bi11	1391	12	1359	10
Bi12	1399	22	1359	21
Bi13	1359	18	1359	18
Bi14	1391	4	1359	3
Bi15	1071	3	Not used	Not used
Bi16	1360	6	1359	6
Bi18	1359	9	1359	9
Bi19	1388	8	1359	6

The analysis generated 946 microsatellite alleles (Table S4). The unbiased haploid diversity ranged from 0 for completely clonal populations, such as 22 and 23 from Portugal, to 0.588 for population 57 from Scandinavia, with a mean of 0.413 (Table 2). Allelic richness (AR) ranged from 0 to 4.0 for the same populations, with a mean value of 2.926, and private allelic richness (PAR) ranged from 0 to 0.214 for population 10 from Morocco, with a mean of 0.027. As expected, PAR values were low in groups of geographically close populations, as from Scandinavia. At a regional level, Scandinavia population showed the highest AR and PAR values, whereas Great Britain the lowest, without private alleles (Table 3). The Alps, Iberia and Carpathian populations contained similar levels of allelic richness, but the Carpathian ones lacked private alleles. The African region, despite the low sampling, was revealed as a place with high levels of private alleles. The most diverse region was north Europe, but close to Central Europe according to allelic richness (Table 3). AR and PAR values for each type of substrate are represented in Table S5. Genetic diversity for specimens on different substrate was difficult to compare due to the unbalanced sampling sizes, but specimens growing on

trunks and twigs contained high levels of private alleles, whereas saxicolous specimens lacked any private allele.

Levels of linkage disequilibrium (rBarD) were usually high, as expected in asexual lichen populations. Significant levels of rBarD were found in 50 of the 64 populations, indicating that the populations have a clonal reproduction. In the remaining 14 populations the presence of sexual reproduction cannot be excluded, but apothecia are not more frequent in these populations than in those with high rBarD levels. While apothecia are most abundant in Scandinavia, rBarD did not have lower levels than in other regions where apothecia are rare or absent. K-test showed signals of recent population expansion in the following 19 localities: 3, 4, 6, 9, 17, 20, 22, 23, 30, 37, 43, 44, 45, 48, 49, 50, 58, 62, and 64.

AMOVA analysis (Table S6) revealed that most of the genetic variation occured within populations (76.81 %), with lower amounts of variations between populations (20.89 %), and insignificant values between the regions shown in Fig. 1 (2.30 %). The permutation test indicates that calculated values are always lower and more statistically significant ($P \leq 0.035$) than the random values, suggesting that differences among populations ($F_{ST} = 0.232$) and regions ($F_{CT} = 0.023$), are statistically significant.

Table 2. Results of the analyses for each population, indicating the number of specimens (n), number of non-clonal specimens, percentage of polymorphic loci, unbiased haploid genetic diversity (uh), unbiased measure of linkage disequilibrium (rBarD, in bold = significant values with a p-value of 0.001), rarefied allelic richness (AR), rarefied private allelic richness (PAR), number of loci with negative values in the K-test (in bold = significant values with a p-value of 0.05), and putative population disturbances (-: well-preserved more or less uniform forest).

Population number	n	Non-clonal specimens	Polymorphic loci (%)	uh mean (stand. error)	rBarD	AR	PAR	K-test	Putative population disturbances
1	20	11	93 %	0.492 (0.058)	**0.300**	2.642 (0.307)	0.014 (0.014)	5	-
2	20	10	79 %	0.336 (0.079)	**0.166**	2.500 (0.402)	0.000 (0.000)	10	-
3	20	18	100 %	0.535 (0.056)	**0.071**	3.642 (0.520)	0.080 (0.071)	**11 expansion**	-
4	15	4	50 %	0.163 (0.050)	**0.371**	1.500 (0.138)	0.000 (0.000)	**11 expansion**	Saxicolous specimens
5	22	16	93 %	0.466 (0.071)	**0.055**	3.142 (0.430)	0.000 (0.000)	10	-
6	21	15	100 %	0.553 (0.046)	**0.125**	3.642 (0.341)	0.098 (0.079)	**11 expansion**	-
7	22	21	100 %	0.486 (0.057)	**0.183**	3.285 (0.450)	0.005 (0.005)	9	-
8	22	14	100 %	0.492 (0.050)	**0.233**	3.500 (0.521)	0.000 (0.000)	8	Apothecia present
9	22	9	36 %	0.127 (0.059)	0.068	1.642 (0.307)	0.076 (0.076)	**14 expansion**	-
10	21	12	86 %	0.440 (0.064)	**0.360**	2.642 (0.414)	0.214 (0.152)	5	-
11	22	12	79 %	0.363 (0.068)	**0.115**	2.285 (0.354)	0.000 (0.000)	9	Sparse and dry forest
12	21	20	93 %	0.411 (0.071)	0.023	3.000 (0.363)	0.000 (0.000)	9	Artificial forest
13	13	11	71 %	0.386 (0.075)	0.053	2.357 (0.324)	0.000 (0.000)	8	Apothecia present

14	19	16	100 %	0.525 (0.038)	**0.224**	3.214 (0.350)	0.003 (0.003)	7	-
15	22	16	100 %	0.559 (0.053)	**0.128**	3.428 (0.571)	0.129 (0.088)	8	-
16	20	20	100 %	0.573 (0.033)	**0.126**	3.285 (0.398)	0.071 (0.071)	4	Some tourism, apothecia present
17	14	6	71 %	0.255 (0.066)	**0.221**	2.071 (0.245)	0.071 (0.071)	**14 expansion**	Apothecia present
18	23	16	100 %	0.519 (0.046)	**0.175**	3.285 (0.398)	0.000 (0.000)	5	Apothecia present
19	23	13	100 %	0.366 (0.053)	0.031	2.642 (0.289)	0.000 (0.000)	10	-
20	9	1	0 %	0.000 (0.000)	NA	1.000 (0.000)	0.000 (0.000)	**14 expansion**	Saxicolous
21	10	1	0 %	0.000 (0.000)	NA	1.000 (0.000)	0.000 (0.000)	**14 expansion**	Saxicolous
22	23	4	71 %	0.276 (0.059)	**0.380**	1.928 (0.195)	0.000 (0.000)	**11 expansion**	Village close, apothecia present
23	23	5	86 %	0.356 (0.057)	**0.183**	2.000 (0.148)	0.000 (0.000)	7	Near pastures
24	9	5	64 %	0.317 (0.079)	**0.258**	2.071 (0.286)	0.000 (0.000)	10	Artificial forest
25	23	14	71 %	0.375 (0.072)	**0.096**	2.214 (0.317)	0.002 (0.002)	7	-
26	23	21	100 %	0.505 (0.046)	**0.268**	3.357 (0.570)	0.000 (0.000)	7	Some tourism, apothecia present
27	23	21	93 %	0.451 (0.063)	0.023	3.071 (0.412)	0.000 (0.000)	8	Human activities and constructions
28	23	18	100 %	0.466 (0.041)	**0.336**	3.214 (0.350)	0.002 (0.002)	6	Tourism
29	23	23	100 %	0.494 (0.057)	**0.144**	3.571 (0.531)	0.008 (0.008)	10	Human constructions, apothecia present
30	22	20	86 %	0.337 (0.072)	0.034	2.857 (0.553)	0.008 (0.008)	**12 expansion**	Apothecia present
31	23	20	100 %	0.056 (0.048)	**0.184**	3.785 (0.612)	0.000 (0.000)	5	Apothecia present
32	21	19	93 %	0.380 (0.068)	0.044	2.642 (0.357)	0.000 (0.000)	7	Apothecia present

33	21	17	79 %	0.362 (0.073)	**0.076**	2.428 (0.309)	0.000 (0.000)	8	Recent fire, tourism
34	22	20	100 %	0.494 (0.063)	**0.224**	3.285 (0.518)	0.070 (0.064)	6	-
35	16	15	86 %	0.364 (0.060)	0.023	2.500 (0.402)	0.000 (0.000)	10	Apothecia present
36	22	21	100 %	0.409 (0.063)	**0.146**	3.357 (0.487)	0.000 (0.000)	10	-
37	24	24	100 %	0.477 (0.059)	**0.056**	3.571 (0.551)	0.069 (0.069)	**12 expansion**	Apothecia present
38	25	23	93 %	0.428 (0.059)	**0.032**	2.642 (0.289)	0.000 (0.000)	5	-
39	21	17	86 %	0.428 (0.059)	**0.058**	2.785 (0.408)	0.008 (0.008)	8	-
40	20	19	100 %	0.480 (0.035)	**0.232**	3.214 (0.394)	0.071 (0.071)	9	Some tourism
41	21	20	93 %	0.399 (0.059)	0.005	2.642 (0.341)	0.000 (0.000)	9	-
42	21	16	100 %	0.405 (0.049)	**0.290**	2.714 (0.411)	0.068 (0.068)	10	-
43	25	19	100 %	0.404 (0.062)	**0.121**	3.000 (0.377)	0.000 (0.000)	**11 expansion**	-
44	20	19	93 %	0.416 (0.072)	0.023	3.142 (0.442)	0.071 (0.071)	**12 expansion**	Tourism
45	25	17	100 %	0.330 (0.053)	**0.163**	3.000 (0.432)	0.000 (0.000)	**13 expansion**	-
46	23	21	100 %	0.481 (0.057)	**0.049**	3.357 (0.487)	0.076 (0.061)	8	-
47	24	22	100 %	0.480 (0.063)	**0.056**	3.642 (0.607)	0.059 (0.059)	10	-
48	23	23	100 %	0.522 (0.056)	**0.081**	3.642 (0.560)	0.186 (0.134)	**11 expansion**	Farm close
49	24	22	100 %	0.519 (0.051)	**0.116**	3.857 (0.404)	0.089 (0.069)	**11 expansion**	-
50	25	24	100 %	0.455 (0.062)	**0.131**	3.285 (0.437)	0.000 (0.000)	**12 expansion**	Apothecia present
51	11	10	100 %	0.549 (0.053)	**0.216**	3.000 (0.347)	0.001 (0.001)	6	Trees on a bog, apothecia present

52	25	25	93 %	0.427 (0.065)	0.024	3.000 (0.444)	0.000 (0.000)	7	Competence with *Bryoria fremontii*.
53	25	22	100 %	0.479 (0.048)	**0.074**	3.000 (0.331)	0.000 (0.000)	8	Close to crops, apothecia present
54	23	19	100 %	0.581 (0.040)	**0.250**	3.642 (0.427)	0.014 (0.010)	5	Apothecia present
55	24	22	86 %	0.388 (0.073)	**0.056**	2.857 (0.442)	0.019 (0.013)	7	-
56	22	21	100 %	0.580 (0.051)	**0.218**	3.571 (0.521)	0.002 (0.002)	7	-
57	24	22	100 %	0.588 (0.037)	**0.204**	4.000 (0.419)	0.017 (0.012)	5	Human activities close, apothecia present
58	21	19	100 %	0.516 (0.052)	**0.103**	3.428 (0.250)	0.000 (0.000)	**11 expansion**	Competence with *Bryoria fremontii*.
59	25	16	100 %	0.493 (0.038)	**0.408**	3.357 (0.414)	0.000 (0.000)	8	Competence with *Bryoria fremontii*, apothecia present
60	24	21	100 %	0.483 (0.058)	**0.312**	3.571 (0.571)	0.000 (0.000)	8	Apothecia present
61	22	18	100 %	0.596 (0.046)	**0.266**	3.642 (0.464)	0.064 (0.064)	4	Apothecia present
62	24	13	93 %	0.286 (0.059)	**0.332**	2.714 (0.338)	0.059 (0.059)	**12 expansion**	Some tourism, apothecia present
63	25	22	93 %	0.330 (0.069)	0.037	2.928 (0.450)	0.000 (0.000)	10	Forest between houses
64	25	14	57 %	0.202 (0.065)	**0.114**	2.071 (0.370)	0.000 (0.000)	**11 expansion**	-

Table 3. Allelic richness (AR) and private allelic richness (PAR) for each region, with standard deviation in brackets.

	Region	specimens	AR	PAR	specimens	AR	PAR
North	Great Britain	32	2.642 (0.439)	0.000 (0.000)	588	7.500 (1.207)	1.357 (0.452)
	Scandinavia	556	7.428 (1.170)	1.357 (0.452)			
Central	Alps	189	5.357 (1.014)	0.357 (0.199)	641	7.071 (1.442)	0.857 (0.274)
	Carpathians	179	5.428 (0.976)	0.000 (0.000)			
	Iberia	273	5.571 (0.976)	0.285 (0.125)			
South	Africa	65	4.571 (0.947)	0.428 (0.227)	130	5.571 (1.087)	0.500 (0.251)
	Mediterranean	65	4.214 (0.575)	0.071 (0.071)			

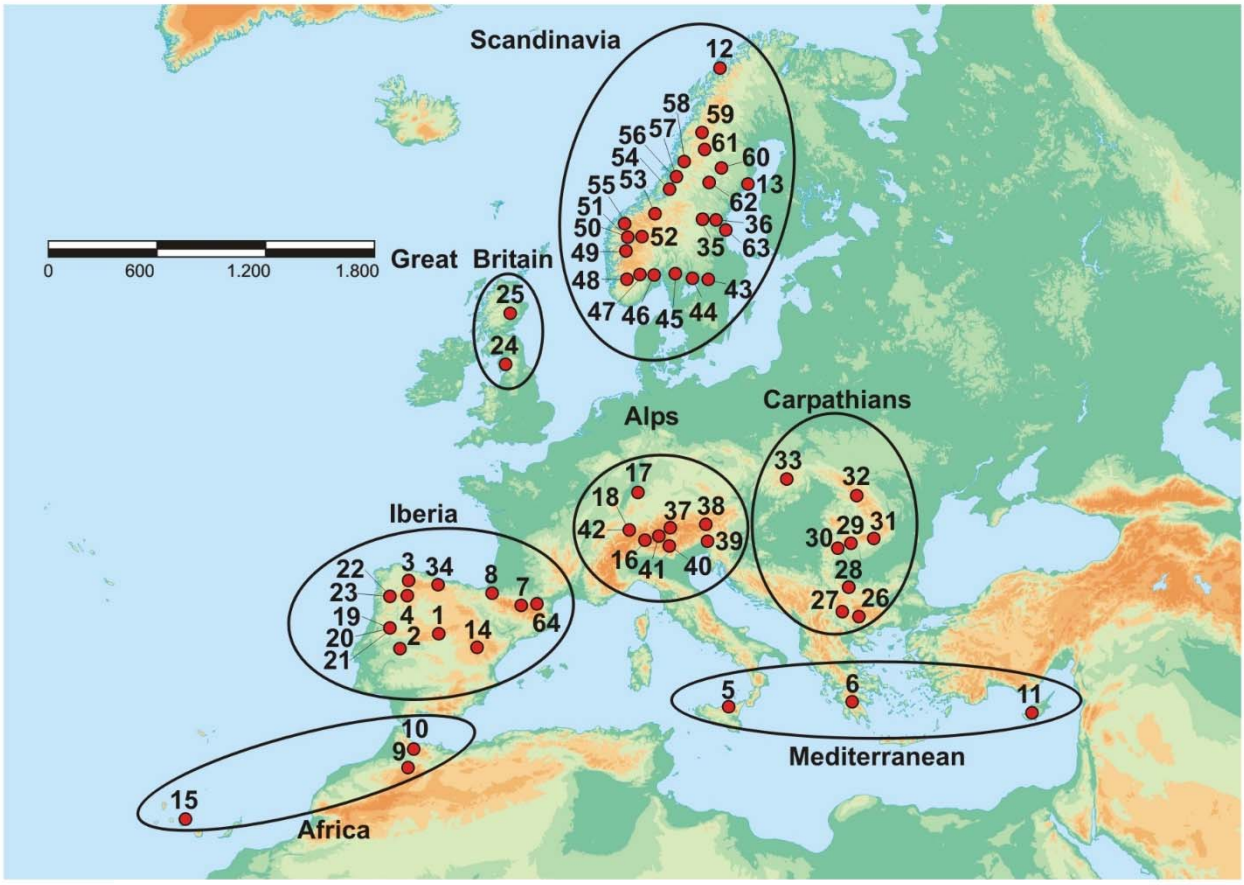

Fig. 1. Map of sampled locations of *Bryoria fuscescens* and regional assignation of each population. Map obtained from All-free-download.com.

Genepool detection

STRUCTURE output (Fig. S1), thorough the ΔK method (Evanno *et al.* 2005), suggests three genepools (*k3* in Fig. S1). The cluster showed in blue forms a well characterized genepool, whereas the red and green clusters contained many intermediate specimens that connect them. DAPC analysis also gives three as the most reasonable number of genepools (DAPC *k3* in Fig. S1), but groups are not exactly coincident in STRUCTURE. Both analyses become uninformative from *k4* longer (Fig. S1), and new putative genepools are formed dividing the same cluster (red and orange respectively). DAPC analysis is more appropriate for species with high probability of linkage disequilibrium or asexual reproduction than STRUCTURE. With the partial support of STRUCTURE, DAPC *k3* was selected as the number of clusters that best fits with our data. Based on the two retained discriminant functions of DAPC, the probabilities of group membership are high (Genepool 1 = 0.99; Genepool 2 = 0.98, Genepool 3 = 0.86). Genepool 1 is equivalent to the blue STRUCTURE cluster. Genepool 2 has both, the red and green clusters of STRUCTURE as expected by the presence of many intermediate specimens. Genepool 3 was not detected in STRUCTURE until *k6*, probably due to the highly unbalanced cluster sizes.

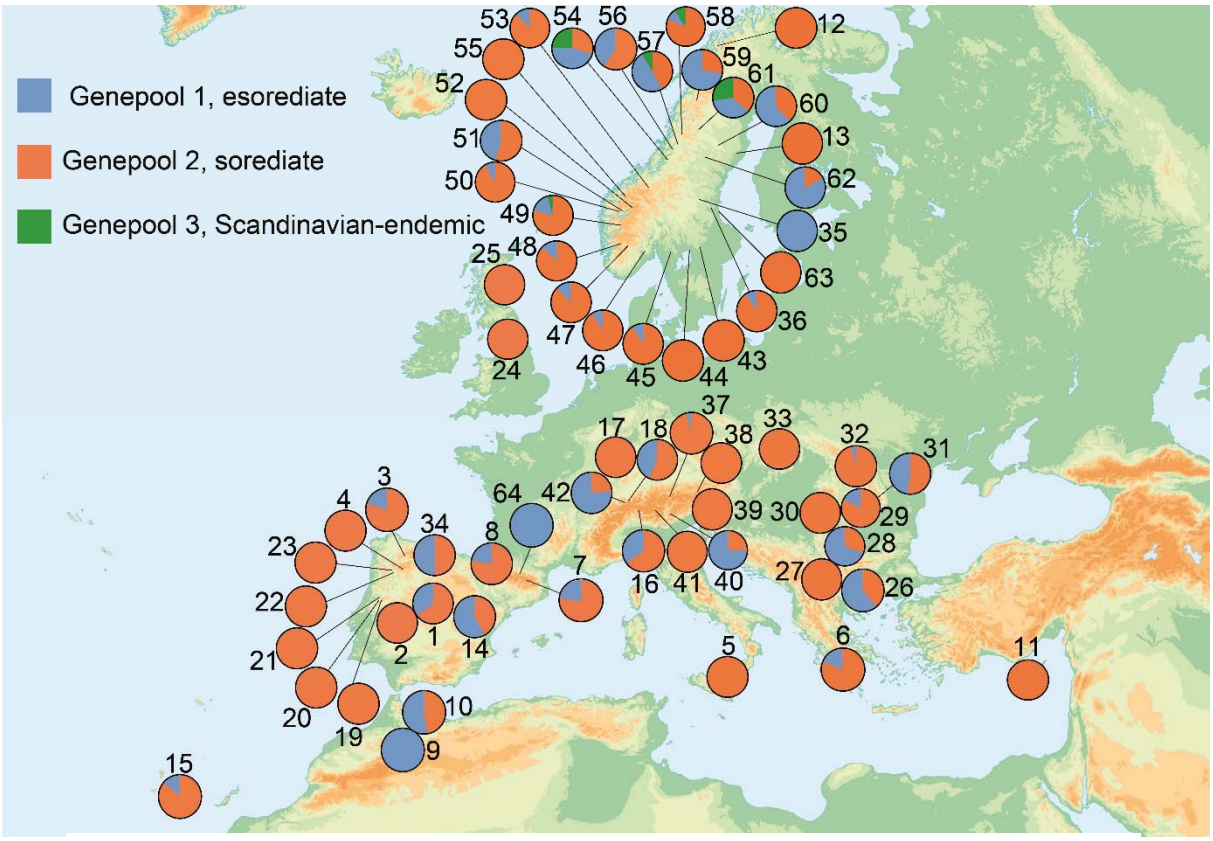

Fig. 2. Distribution of the genepools predicted by DAPC analysis across all the studied area. Numbers refers to population identifier.

Genepool phenotypes

Morphological and chemical information of the specimens (according to DAPC), and its genepool assignation, can be seen in Table S7. Genepool 1 (blue) contains 330 specimens lacking soralia and fumarprotocetraric acid, and although other extrolites can be present, alectorialic and barbatolic acids are common. Genepool 2 (orange), with a high genetic diversity, contains 1008 specimens from all known phenotypes. Genepool 3 (green) contains only 18 specimens characterized by its uniformity, with only alectorialic and barbatolic acids and without soralia. No other relation among genepools and the studied morphological characters was detected. Genepool 1 is composed by 287 (87 %) *capillaris* morphs and 44 (13 %) *fuscescens*, Genepool 2 by 130 (13 %) *capillaris* and 878 (87 %) *fuscescens,* and Genepool 3 is only composed by *capillaris* morphs. The lichenicolous fungus *Raesaenenia huuskonenii* had arbitrary distribution among the genepools, morphological characters, and extrolite composition, but was not detected in Genepool 3.

Potential distribution analyses

Distribution of the genepools in the studied area (Fig. 2) revealed Genepool 3 (green) as restricted to north Scandinavia, whereas 1 (blue) and 2 (orange) appear as overlapping over all the area and lack any differential distribution pattern. Genepool 2 was not detected in Cyprus, Great Britain, Portugal or Sicily, but its absence could be attributable to the lower sampling of this regions. No specimens of Genepool 1 have been detected in saxicolous populations (populations 4, 20 and 21).

To detect putative selective environmental adaptations of the genepools showed by the SSRs data, a potential distribution of each cluster was estimated using MaxEnt. Using the sampled points to estimate the preferred environmental conditions, the probability of adequate conditions was predicted over all the studied area. The R package MaxentVariableSelection analysis was used to discard the non-informative variables, resulting in the selection of BIO1, BIO 2, BIO 4, BIO 8, BIO 9, BIO 10, BIO 11, BIO 12, BIO 15, BIO 18, and BIO 19. Analyses using the selected variables were not significantly different than those in which all variables were included. AUC values for clusters 1, 2, and 3 were 0.98, 0.98, and 0.96 respectively. Models with AUC values between 0.9–0.97 can be considered very good and between 0.97–1 excellent, supporting the predicted distribution. Results presented in Fig. S2 show that *Bryoria fuscescens* is generally absent in the lowlands, with the exception of the hyper-oceanic regions of Norway. Some un-sampled regions were predicted as suitable for *Bryoria fuscescens*, such as the Lebanese mountains, Turkey, the Ethiopian mountains, the Balkan Peninsula and the Caucasus mountains. There are some herbaria records of this species from those regions (www.gbif.org), supporting the distribution prediction. The potential distribution

map predicts an unexpected absence of *Bryoria fuscescens* from the lowlands of the boreal region, from Sweden to Russia, while there are numerous records from these areas (Myllys *et al*. 2011). These regions may however have different environmental conditions from those of our sampling points so that the analysis indicates these areas as unsuitable for *Bryoria*. Moreover, it is conceivable that *Bryoria* could inhabit micro-niches, which are not present in the bioclimatic layers.

No significant differences in the potential distribution of cluster 1 and 2 are evident. Genepool 3 shows a distinct potential distribution, centered mainly in the oceanic parts of Norway, southern Iceland, and the Alps. The four most significant bioclimatic variables participating in the prediction were in order of importance; cluster 1: BIO10, BIO8, BIO1, and BIO19; cluster 2: BIO10, BIO1, BIO19, and BIO8; cluster 3: BIO2 (double than the next one), BIO15, BIO8, and BIO18.

Analyses on past bioclimatic maps (Fig. 3) showed significant potential distributions during the Last Inter-Glacial period (around 130 000 years ago (ya)), when the climate was warmer than today. Potential distribution was reduced in Southern Europe and especially in the southeast: Balkans, Carpathians, Turkey, or Caucasus. Apart from Iceland, no new suitable land extensions are shown. Potential distribution evidently suffered the biggest alteration during the Last Glacial Maximum (22 000 ya), where lower sea levels produced land bridges between the European peninsulas and islands to the continent. Ice sheets covered great parts of north and central Europe, and the distribution is shown as southern and at lower levels. No adequate habitats seem to have been present in Scandinavia, while the west coast of Central Europe shows great extensions of appropriate regions. Northern and southern lowlands of the Alps are also showed as possible glacial refugia. During the Mid-Holocene (6 000 ya) potential distribution is very similar to that today, but slightly more extensive.

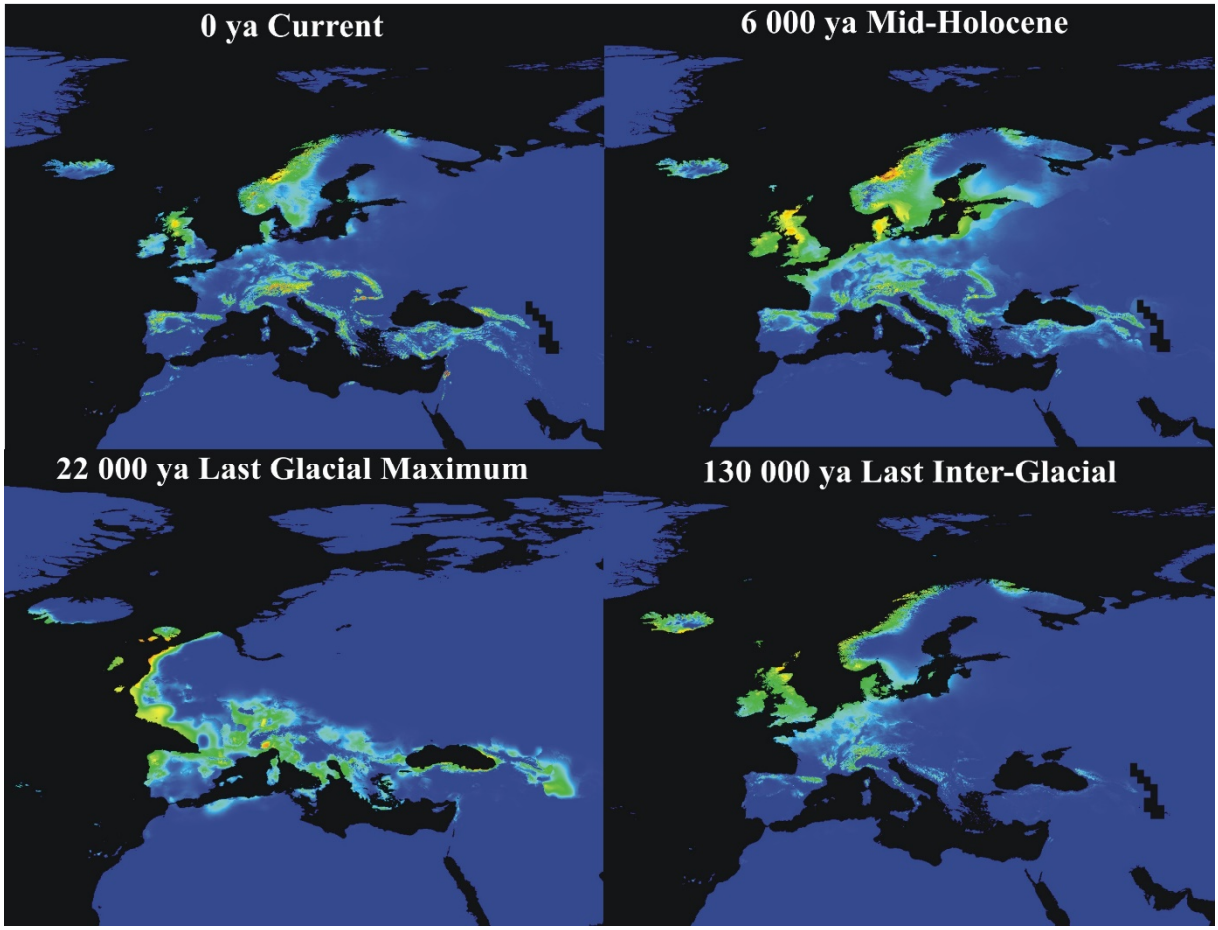

Fig. 3. Potential distribution maps predicted by Maxent. ya = years ago**.**

The potential distribution of the major chemotypes (Fig. S3), suggested different climatic preferences for some of them. Specimens with barbatolic or fumarprotocetraric acids had a similar preference as the whole species. Specimens with gyrophoric acid however had a boreal preference, being rare in southern regions; the Atlantic Scandinavian coasts were predicted as significantly suitable for this chemotype. Norstictic acid specimens were indicated as preferring the boreal oceanic regions rather than the southern mountains, but psoromic containing specimens were mainly centered in the great mountain ranges of Central Europe.

Genetic-geographic analyses

The isolation by distance analysis (Fig. 4) reveal a positive correlation in Cluster 2 (sorediate genepool, r = 0.246, *P* = 0.001), but this correlation was not so clear in Cluster 1 (non-sorediate genepool, r = 0.147, *P* = 0.054). This indicates a continuous but low cline of genetic isolation in the distance for genepool 2, but this is not so evident in genepool 1. Whereas in genepool 1 dots are mainly below a genetic distance of 0.4, in genepool 2 they

appear much more dispersed and centered around 0.4. Genepool 2 shows higher levels of genetic isolation among populations, but this isolation does not increase significantly in geographically distant locations. The most distant pairs of populations are not the most genetically isolated, and the most genetically isolated are found at various geographical distances.

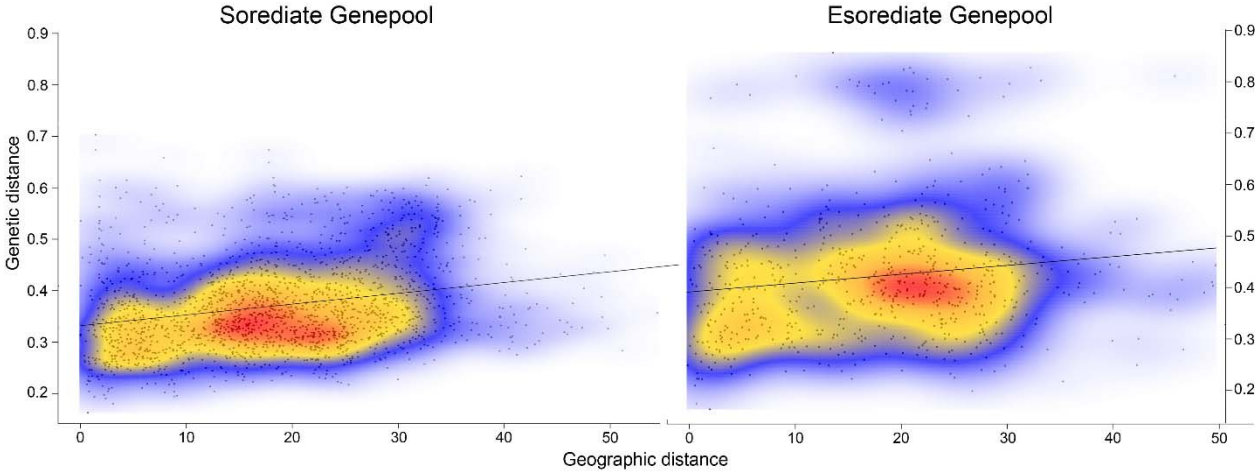

Fig. 4. Isolation by distance plots for Genepool 1 (esorediate genepool, r = 0.147) and Genepool 2 (sorediate genepool, r = 0.246).

The estimation of the migration rates (M) among 38 tested putative migration routes, and the effective population size (Θ) of the recipient populations are shown in Table S8. Comparisons of migration intensity among regions using the 2Nm value (M × Θ) are shown in Fig. 5. Main migration events are focused between Scandinavia and Central-Southern Europe. Scandinavia is revealed as the region with highest emigration levels.

Phylogenetic analysis

Of the 1359 analyzed specimens, 34 representing the wide geography and phenotypical variability of the species were selected for DNA sequencing (table S2). A phylogenetic reconstruction using the loci ITS, IGS and GAPDH and the same samples used in Chapter 4 for the revision of *Bryoria* sect. *Implexae*, revealed that specimens L54.19, L54.20, and L58.08 belong to the cryptic species *Bryoria pseudofuscescens*, whereas all the other samples matched with *B. fuscescens* (Fig. S4). Those three samples belong to Cluster 3, but L58.17, the fourth sequenced sample of this cluster, belongs to *B. fuscescens*. Locus GAPDH, the most informative in the identification of species within this species aggregate, had no successful amplifications in Cluster 3 even after repeating the DNA extraction three times and with different PCR conditions.

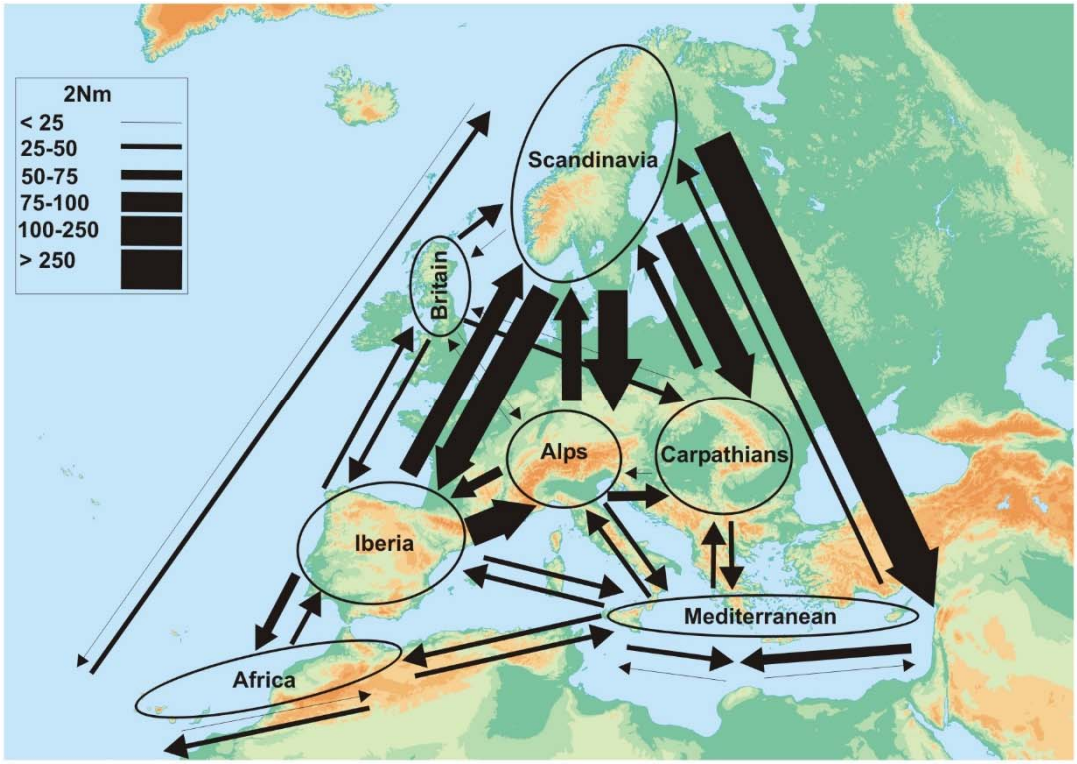

Fig. 5. Estimate of migration patterns inferred from Migrate analysis. Arrows represent the numbers of immigrants per generation (2Nm = M x Θ). Arrow width is proportional to the degree of immigration into a region.

Phylogenetic reconstruction of the eight loci (Fig. S5) revealed a congruent topology among the ITS, IGS and GAPDH regions, whereas each flanking region (the five FRBi loci) showed differing topologies. The three genepools predicted by both STRUCTURE and DAPC analysis are not shown as monophyletic, although in some loci there is a trend to form lineages mainly composed by one genepool.

Algal ITS sequencing revealed that all samples contained *Trebouxia simplex* group as the algal partner. Two lineages can be distinguished, one for the Cluster 1 and 2 (with five haplotypes) and the other for the Cluster 3 (with two haplotypes). Specimen L58.17 contained both lineages, as revealed by the presence of a double lecture of equal intensity in the electropherogram after repeat the PCR two times.

Discussion

Population dynamics

Bryoria fuscescens agg. comprises asexual lichens rarely producing functional apothecia. According to our results, and despite its predominantly clonal reproduction, genetic variability cannot be considered low, and completely clonal populations were not detected, with the exception of localities 21 and 22. Further, saxicolous populations could have originated from a recent colonization event from a single specimen that clonally invaded the outcrop (as suggested by the K-test). All populations over rock showed recent expansion signals and low levels of genetic diversity. Saxicolous habitats, due to their reduced and isolated nature in the studied regions, seem more liable to extinction events than epiphytic ones, impeding the formation of genetically structured populations. Saxicolous specimens are more frequent in arctic regions, but unfortunately we were unable to include these in our study, where a more varied population dynamic might be expected. Clonally levels were fewer in the Scandinavian peninsula, where this species is frequent and apothecia are more common. The lowest levels of genetic diversity were found in Portugal, and unexpected particularly in populations 22 and 23, composed of large specimens with apothecia. This could be explained by recent colonization events, as the region has been adversely affected by past human activities, and consequently local *Bryoria* populations could be extinct. Private alleles appear in isolated populations, as in the Canary Islands, Morocco, and Greece, where geographical barriers may impede newly originating haplotypes spreading to other parts of Europe. On the other hand, some isolated populations, as those from Sicily, Cyprus and Great Britain, do not contain exclusive alleles, and could have originated by continental colonization events. Some populations included in clusters, as numbers 3, 16, 17, 34, 37, 40, 42, 46, 47, 48, 49, 61 and 62, contained private alleles, especially those from the Alps or southern Scandinavia, indicating high genetic richness in this regions. Populations with a putative recent expansion were also detected. These more probably occur in human-affected areas or after an environmental catastrophe, but expansion events are not correlated with those activities, at least as observed in the field without any information of the recent history of the localities.

Forest overexploitation by humans could explain the local extinction of *Bryoria* populations, as in the case of some regions of Portugal. But in many populations where *Bryoria* coexists with current human activities, as tourism, roads, or artificial forest margins, allelic richness is not lower than the average. *Bryoria* needs relatively high light levels to grow, which cannot be found on dense mature forests. Roads or unintentional forest clearing typical of human-visited places may increase light levels and habitat heterogeneity, leading to more complex structured lichen communities. Populations collected close to a road usually

contained denser lichen communities on the road margins than inside adjacent forests, especially in dense forests. Lichens usually are very sensitive to an excess of nutrients, nevertheless, unpaved dusty roads or isolated trees on nitrate-enriched pastures, seems not to have negatively effects in adequate rainy *Bryoria* habitats. Sometimes the *Bryoria* apparent to be favoured by this extra nutrients, especially in very humid places. Populations such as 27, 29, 39, 48, 53, and 57, in which human nutrient contribution seems evident, contains higher allelic richness than the average.

Localities with apothecia do not show higher genetic diversity than localities without, suggesting that sexual reproduction is not occurring or is not important in the production of genetic diversity. Moreover, no spores were seen after an examination of the apothecia of collected specimens. Twenty populations contained apothecia, but linkage disequilibrium analysis predicted clonal reproduction for all except four. Putative sexual reproduction was detected also in nine non-apotheciated populations, indicating there is no correlation between presence of the apothecia and sexual reproduction as detected as free recombination of the loci.

Bryoria fuscescens appears along Europe forming isolated populations, especially in southern areas. With this island-like distribution it might be expected that geographic distance would act as a dispersal barrier, but distant populations are not significantly more isolated than closer ones (Fig. 4). Ascospores, given apothecia rarity, and their doubtful functionality, are evidently not the main dispersal factor. Two major genepools are detected on *Bryoria fuscescens*, one with soralia, producing dispersal propagules of restricted range (Walser *et al*. 2001), and another without detected propagules. Dispersal capacities are similar in both genepools, showing the reduced contribution of the soredia. Lichen fragments may act as dispersal propagules if are helped by animals like birds, but this seems not enough to explain this highly connected populations. A lack of isolation by distance does not means necessarily that *Bryoria* has excellent dispersal capacities, but may be an artefact produced by ancestral widely distributed haplotypes and slow genetic drift, as suggested for the lichens *Cavernularia hultenii* and *Cetraria aculeata* (Printzen & Ekman 2002; Printzen *et al*. 2003; Fernández-Mendoza *et al*. 2011). *Bryoria fuscescens* is a relatively young species with an estimated origin around 100.000 years ago (Chapter 4), suggesting that some ancestral haplotypes could be currently present in distant geographical regions. Because of its recent origin, it may colonize distant regions quickly, indicating in any case some dispersal capacities. Probably, a mix of wide distributed ancestral haplotypes together with better dispersal capacities than expected even for lineages not producing any know propagule, may explain the apparent absence of genetic isolation by distance.

Phylogeography

Europe has been strongly affected by glacial events, influencing main phylogeographic patterns of species living here. The origin of *Bryoria fuscescens* around the end of the last interglacial period (Chapter 4), suggests that the current genetic distribution could be strongly influenced by the last glacial events. A single unique attempt to detect European glacial refugia on lichens was performed in *Lobaria pulmonaria* (Widmer *et al*. 2012), suggesting that apart from Italy and the Balkans, the area west of the Ural Mountains may also important. During the Last Glacial Maximum (LGM), around 22 000 ya, ice covered Scandinavia, the Alps, and the greater part of Great Britain and Central Europe. European ice cover had an asymmetrical border, southerly in the more humid west, and fuller north in the drier east (Tzedakis *et al*. 2013). In our study, LGM potential distribution maps show a preference of *Bryoria* to Atlantic oceanic coasts rather to the western Europe continental regions. Denser potential regions (red and yellow in Fig. 3) are detected from Scottish to Irish coasts, the north-west Iberian Peninsula, the lowlands around the Pyrenees, northern and especially southern lowlands of the Alps and southern and eastern coasts of the Black Sea. This match with the high levels of private alleles detected in Alps and Iberia, but not with the huge Scandinavian richness and low Great Britain diversity. The Great Britain may be expected to contain the remnants of high genetic diversity harboured during the LGM, but this is not observed in our study. The strongly recent or historical human interactions with the Great Britain forests, including air pollution, together with the low and probably not most appropriate collections performed here, may impede to detect this diversity. Genetically rich areas typically indicate glacial refugia (Petit *et al*. 2003), but the richest area, Scandinavia, was covered by ice during the LGM. Scandinavian diversity could come from a migration of the coastal Atlantic refuged specimens to the current oceanic Scandinavian coasts during the climate warming. This correlates with the absence of private alleles between Scandinavia and Great Britain. Nevertheless, recent studies are revealing the importance of northern plant glacial refugees (Tzedakis *et al*. 2013), which could have played a major role on boreal lichens. During the LGM some coastal regions of Scandinavia were ice-free even in northern areas than the arctic circle (Tzedakis *et al*. 2013). Moreover, the *Bryoria* may survived in nunataks (Stehlik *et al.* 2002; Holderegger & Conny 2008), in a similar way as seen currently in Canada and Greenland (Brodo & Hawkswort 1977). These regions seem not present in the past bioclimatic maps used for potential distribution estimation. Then private alleles originated during the LGM in Scandinavian refugia would be mixed with those coming from the putative southern coastal migration, constituting to the high levels of the current Scandinavia. A possible colonization of Scandinavia from Russia seems less probable as past bioclimatic maps do not detect suitable habitats here, but Russian samples would be needed to evaluate the eastern contribution to the Scandinavian

recolonization. Regions as east Europe or Carpathians are detected as non-adequate for *Bryoria* during the LGM, probably due to the dryer environment. This may explain the absence of Carpathian exclusive alleles, as all its haplotypes can be found in other regions. Then Carpathians would be recently colonized from other regions.

Main migrations routes are found between Scandinavia and central-southern Europe (Fig. 5), especially from north to south. Another minor trend is observed from Iberia to Alps and Carpathians. This is agreeing with the hypothesis of a glacial refugia on the Atlantic coastal region (Fig. 3), as haplotypes originated in the refugia should migrated north to Scandinavia, east to Central Europe, and south to Iberia, producing a false appearance of high migration levels between these zones. An unexpected strong migration occurs from Scandinavia to Mediterranean. This may be explained by non-sampled areas intervening, like the Black Sea southern mountains and Caucasian mountains. Haplotypes moved from that regions to the Mediterranean, Carpathians and Scandinavia, as would be shared, may produce a non-real migration detection between this three zones. The absence of private alleles in Sicily and Cyprus may indicate a recent colonization in these islands, whereas Greece maintained its ancient haplotypes merged with the new arrived. Migration rate detected from Cyprus to Greece may support to a Cyprus colonization from eastern regions. Glacial events seem to have played a minor role in Africa, which contain high levels of private alleles and low migration events are detected.

In conclusion, an eastern and western recolonization of Europe, in addition to some Scandinavian refugia, may produce shared haplotypes that could be detected as migration events within these regions. In addition, high level of migration routes could be an artefact of shared ancestral polymorphisms, as demonstrated for other organisms (Muir & Schlötterer 2005).

Evolution

Bryoria fuscescens is a variable and recently originated species with two main morphologies, in the past treated as distinct species, the *fuscescens* and *capillaris* morphs. These morphs are also present in the sister cryptic species *Bryoria pseudofuscescens* from North America, and probably also in the few-studied *B. kockiana* (Chapter 4), suggesting that morphs are more ancient than accepted species divergence. These morphologies are apparently environmentally-independent, as can grow intermixed (Chapter 4). Our data shows here that these morphologies are not genetically fixed in clades or genepools, but a major tendency can be found. Among the studied area three main genepools are detected. Genepool 1 is composed by an 87 % of *capillaris* and 13 % of *fuscescens* specimens, including for example all the samples from the African population 9, which has a very typical *fuscescens*

phenotype. Cluster 2 is the most variable and abundant, with an 87 % of *fuscescens* specimens and 13 % of *capillaris* morphs mainly from the Scandinavian region. Genepool 3 only contains *capillaris* phenotypes. Apart from the major trend to detect *capillaris* in Genepool 1 and *fuscescens* in Genepool 2, there is a trend to include southern *fuscescens* into genepool 1, and northern capillaris into Genepool 2. Moreover, genepools are linked to other phenotypical characters, as the absence of soralia (except in two individuals) and fumarprotocetraric acid (except in three individuals) in Genepool 1, indistinctly produced in genepool 2. Then, Genepool 1 is mainly composed by *capillaris* specimens lacking fumarprotocetraric acid and soralia, while Genepool 2 contains the more variable *fuscescens*. No other of the nine studied morphological characters matched consistently with the microsatellite genepools. Four specimens were sequenced from Genepool 3, three of them showing clonal ITS and IGS sequences with an American *B. pseudofuscescens* specimens, whereas one had clonal sequences with previously studied *B. fuscescens* (Fig. S4). Many populations contained both morphs growing admixed, but despite the major tendency, sometimes morphs were gathered under the same genepool, especially in Scandinavia.

Here we discard that *Bryoria* morphotypes or traditional species are linked to exclusive genepools, as well as that genepools do not conform monophyletic lineages when DNA sequences are used, even in those from the microsatellites flanking regions (Fig. S5). Of the 8 markers used for the phylogenetic reconstruction, only three (ITS, IGS and GAPDH) showed a congruent topology, even though ITS and IGS were few informative. Each microsatellite flanking region marker had its own topology. Incongruent topologies may be the result of various processes, such as paralogy, incomplete lineage sorting, hybridization, or recombination. It is not expected that recombination in haploid asexual species would produce this pattern. In the case that flanking regions markers were multicopy loci, different paralogs may be obtained from different samples. In that case microsatellites of each flanking region would be also multicopy, and double or multiple peaks should be expected in the electropherogram. As microsatellites genepools are morphologically defined, and no more than an expected level of multiples peaks have been detected, we do not expect that paralogy is playing a significant role here. Our topologically inconsistent phylogenetic trees seem compatible with the hypothesis of incomplete linage sorting (ILS) or hybridization among genepools, among *fuscescens* and *capillaris* morphs, and even probably between *Bryoria fuscescens* and *B. pseudofuscescens*. Because sexual reproduction seems absent or extremely rare, incomplete lineage sorting can be playing a major role. According to this, and as may be expected for recently diverged species like this, *Bryoria fuscescens* agg. may be an asexual speciating group largely managed by genetic drift, leading to high levels of ILS of ancestral and recent polymorphisms (Takahashi *et al.* 2001; Jakob & Blattner 2006). Four main

phenotypically well visible characters seems may be influenced by a putative speciation process, the fumarprotocetraric and barbatolic acids production, the formation of soralia and the thallus colour, which is related with the amounts of epicortical substances (Boluda *et al*. 2014). Whereas the production of fumarprotocetraric and barbatolic acids seem lineage-related, production of other extrolites as gyrophoric, norstictic or psoromic may be environmentally dependent (Fig. S3) and not linked to genetic lineages.

When one species suffers a rapid phenotypic speciation or at least faster than the genes evolution, then the apparition of ancestral shared polymorphisms is expected (Muir & Schlötterer 2004). If genes used to study the evolution of one lineage contains ILS or share ancestral polymorphisms, analyses will support the non-monophyly of the clades even if are truly monophyletic. In *Bryoria fuscescens* agg. standard barcoding loci as ITS and IGS cannot distinguish with confidence the three accepted species, and only a more variable region as GAPDH can reveal supported clades, but these clades seems more related with geography than phenotypes. Microsatellites evolve much rapid than DNA sequences, increasing the probability to track ongoing speciation events (Sunnucks 2000). Then microsatellites can reveal phenotypic evolving tendencies masked by DNA sequences with ILS. Phylogenetic incongruence among chemotypes, morphotypes and phylogenies, as well as the apparent low levels of isolation by distance, high levels of migration events among distant areas, and even the detected sexual reproduction signals, could be explained by shared ancestral and recent polymorphisms and ILS. *Bryoria* may have not so good dispersal capacities and sexual reproduction as analyses suggest. Moreover, genetic differentiation among the three *Bryoria fuscescens* agg. species could correspond to a geographical isolation rather than a speciation process, as speciation would be occurring between *capillaris* and *fuscescens* morphs probably at worldwide level or at least in Europe. Genetic diversity associated to neutral markers, like those used here, may not correspond well with markers associated to phenotypes related with life-history (Bekessy *et al*. 2003; Gómez-Mestre & Tejedo 2004). Neutral markers are useful for study gene-flow patterns, as population dynamics, but adaptive markers give the potential evolutionary forces that could produce a speciation process. Perhaps a phylogenetic reconstruction using phenotypically-related loci, as those that are involved in the production of barbatolic or alectorialic acids, epicortical substances and soralia, may reveal phenotypically characterized clades. In summary, our results allow us to hypothesize that *Bryoria fuscescens* agg. is still under a speciation process.

Acknowledgements

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Supplementary material

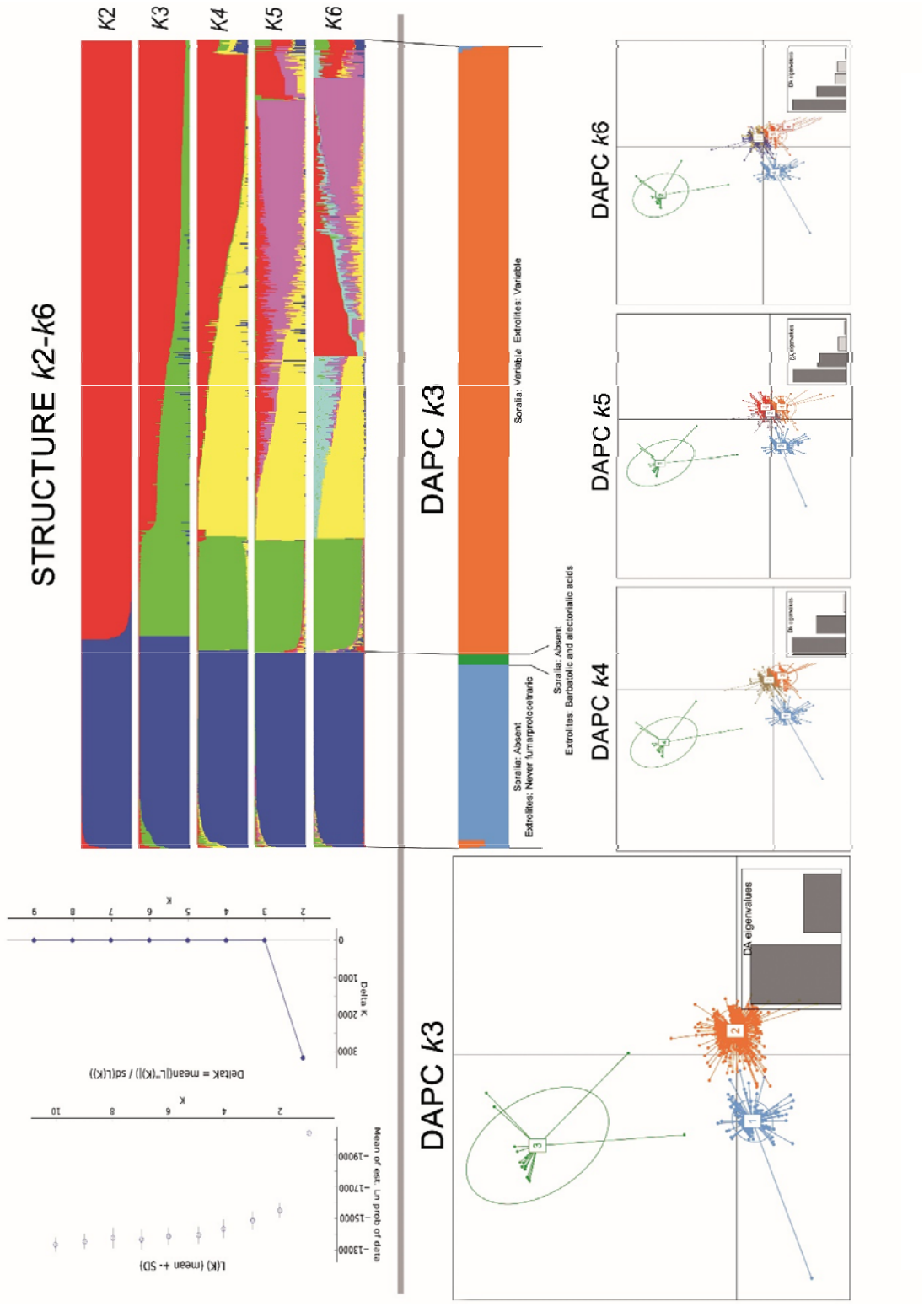

Fig. S1. Up right, STRUCTURE results from k = 2 to k = 6. Up-left, STRUCTUTE-Harvester analysis output, showing k = 3 as the most probable number of clusters. Down, DAPC results from k = 2 to k = 6, with a bar showing the concordance between DAPC k = 3 and STRUCTURE clusters. DAPC k = 3 has been selected as the better clustering method that adjust to our data, indicating in the bar the characteristics of the specimens included in each genepool.

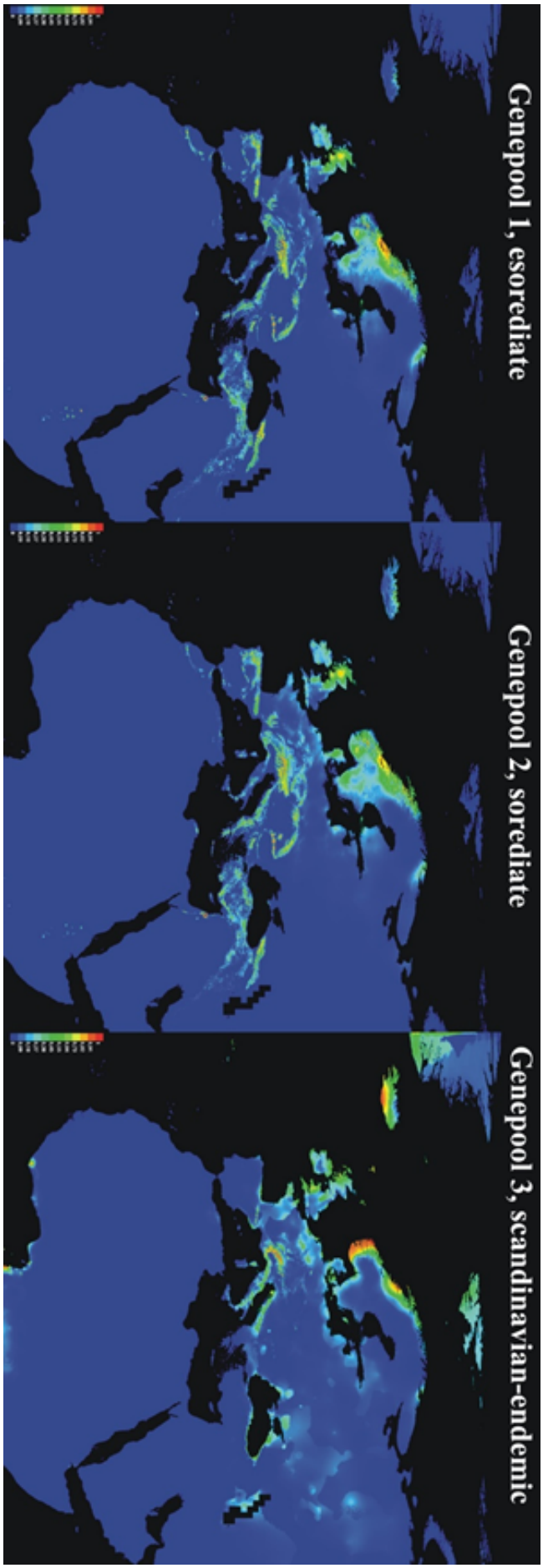

Fig. S2. Potential distribution prediction according to Maxent using the bioclimatic variables: BIO1, BIO 2, BIO 4, BIO 8, BIO 9, BIO 10, BIO 11, BIO 12, BIO 15, BIO 18, and BIO 19.

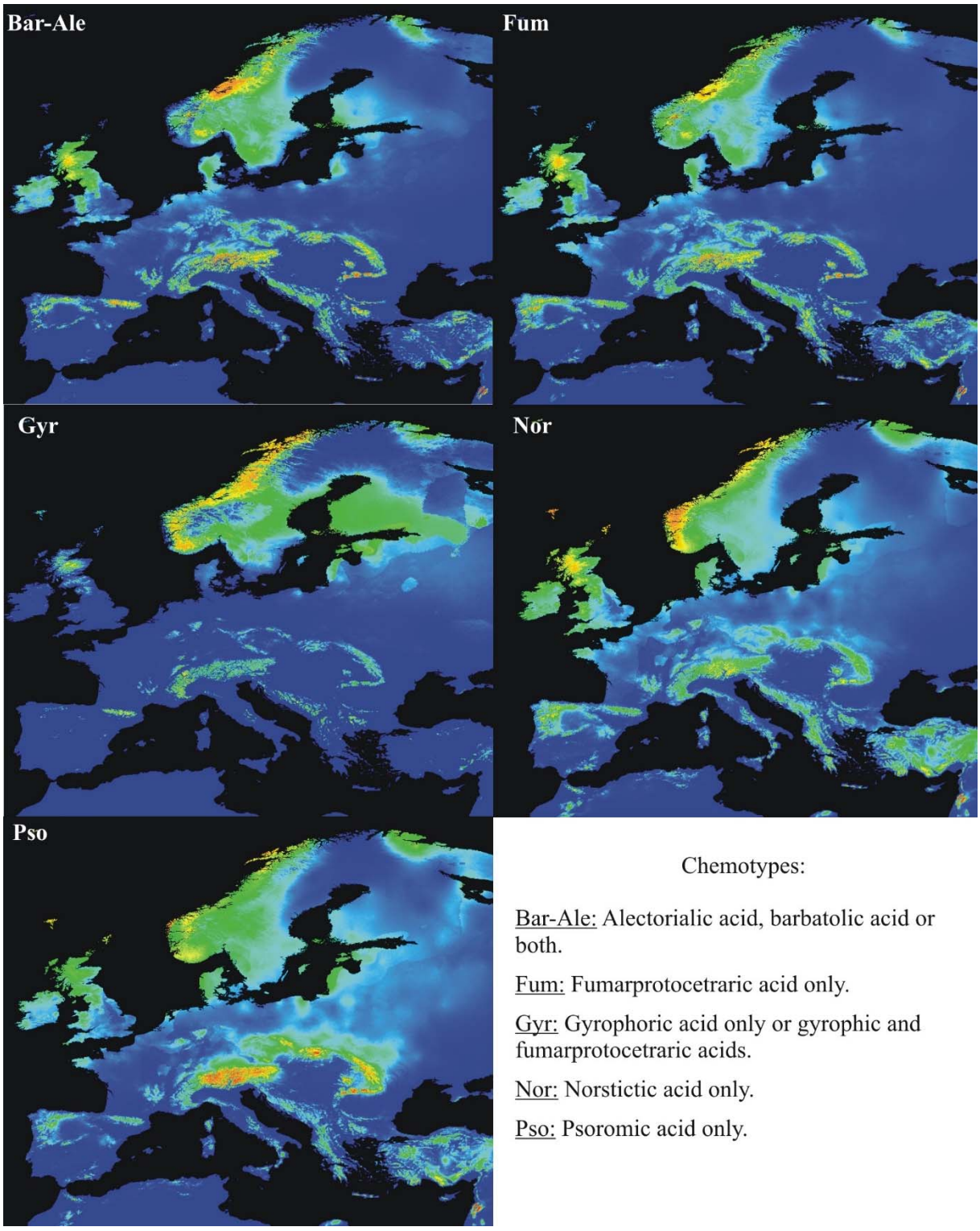

Fig. S3. Potential distribution prediction of the major *Bryoria* chemotypes according to Maxen using the bioclimatic variables: BIO 1, BIO 2, BIO 4, BIO 8, BIO 9, BIO 10, BIO 11, BIO 12, BIO 15, BIO 18, and BIO 19.

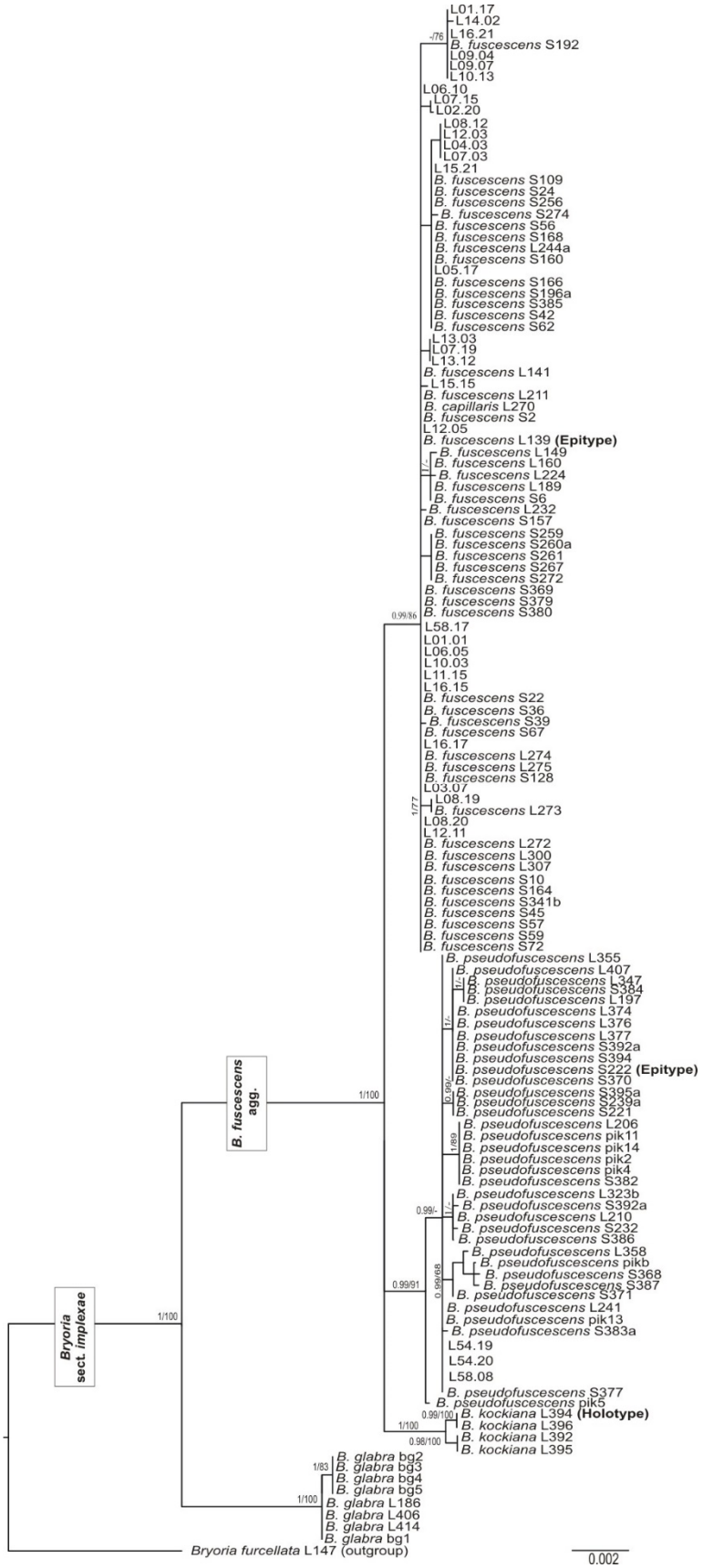

Fig. S4. Bayesian phylogenetic tree reconstruction of *Bryoria* sect. *Implexae* with a concatenated DNA matrix of ITS, IGS and NADH loci.

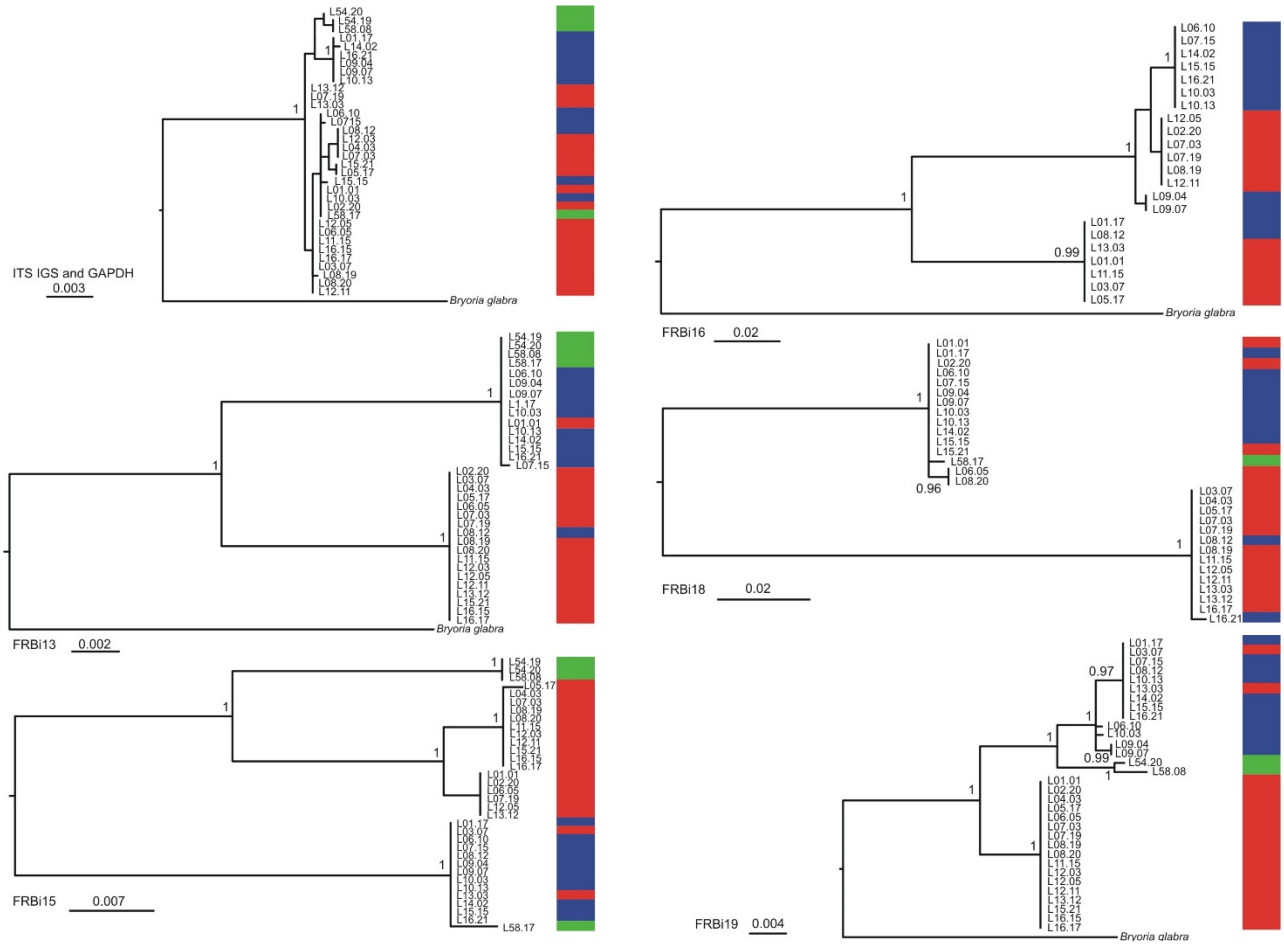

Fig. S5. Bayesian phylogenetic tree reconstruction of the 35 selected specimens using a concatenated DNA matrix of ITS; IGS and GAPDH, and the 5 microsatellites flanking regions FRBi13, 15, 16, 18 and 19. DAPC genepool assignation for each specimen is indicated in colour. Genepool 1 = blue, Genepool 2 = red, Genepool 3 = green.

Table S1. Information of the 64 *Bryoria fuscescens* populations sampled for this study.

Population code	Number of samples	Country, region	Coordinates	Altitude (m)	Habitat
1	20	Spain, Segovia	40°47'34.97"N/03°59'12.62"W	1862	*Pinus sylvestris* forest
2	20	Spain, Cáceres	39°28'38.59"N/05°22'39.98"W	1163	*Quercus pyrenaica* forest
3	20	Spain, Asturias	43°02'22.70"N/06°42'47.60"W	1276	*Betula pubescens* forest
4	15	Spain, Zamora	42º09´50.40"N/06º44´10.90"W	1641	Granitic outcrops
5	22	Italy, Sicily	37°53'22.00"N/14°32'20.20"E	1436	*Quercus* spp. dense forest
6	21	Greece, Peloponese	37°38'16.11"N/22°14'48.41"E	1469	*Abies cephalonica* forest
7	22	Spain, Lérida	42°38'30.00"N/00°58'14.87"E	1908	*Pinus uncinata* forest
8	22	Spain, Navarra	42°58'51.50"N/01°06'32.98"W	1193	*Fagus sylvatica* dense mixed forest
9	22	Morocco, Middle Atlas	33°24'56.09"N/05°05'19.88"W	1915	*Cedrus atlantica* forest
10	22	Morocco, Rif	34°58'03.22"N/04°43'39.20"W	1652	*Cedrus atlantica* forest
11	22	Cyprus, Troodos	34°56'56.61"N/32°50'59.14"E	1617	*Pinus nigra* open forest
12	23	Norway, Troms	69°41'N/18°57'E	100	*Picea abies* plantation
13	14	Sweden, Vasterbotten	63°47'19.08"N/20°20'54.06"E	45	*Picea abies* forest
14	22	Spain, Teruel	40°30'13.78"N/01°39'13.21"W	1670	*Pinus sylvestris* forest
15	22	Canary Islands, Tenerife	28°20'54.94"N/16°30'38.44"W	1384	*Myrica faya*, *Erica arborea* and *Pinus canariensis* forest.
16	21	Switzerland, Graubünden	46°28'29.20"N/09°39'01.60"E	1817	*Pinus cembra* and *Picea abies* forest
17	14	Germany, Baden-Württemberg	48°47'41.70"N/08°45'07.44"E	475	Mixed forest
18	23	Switzerland, Schwyz	47°00'43.86"N/08°44'11.02"E	1494	Mixed coniferous forest
19	23	Portugal, Beira Alta	40°19'22.46"N/07°34'22.43"W	1548	*Pinus nigra*, *P. sylvestris* and *Betula pubescens* open forest
20	9	Portugal, Beira Baixa	40°18'58.26"N/07°33'47.94"W	1576	Granitic outcrops
21	10	Portugal, Beira Baixa	40°19'21.41"N/07°36'07.85"W	1873	Granitic outcrops
22	23	Portugal, Alto Douro	42°00'10.40"N/08°09'50.87"W	755	*Quercus pyrenaica* forest
23	23	Portugal, Alto Douro	42°02'56.64"N/08°10'04.55"W	983	Small *Quercus pyrenaica* tree group
24	9	United Kingdom, England	54°34'55.95"N/03°13'17.32"W	517	*Larix decidua* plantation
25	23	United Kingdom, Scotland	57°00'19.99"N/03°23'32.68"W	363	*Larix decidua* and *Pinus sylvestris* forest
26	23	Bulgaria, Blagoevgrad	41°46'54.29"N/23°26'58.21"E	1727	*Picea abies* dense forest
27	23	Macedonia, Eastern Macedonia	42°02'27.81"N/22°21'06.85"E	1626	*Fagus sylvatica* human-disturbed forest
28	23	Bulgaria, Montana	43°11'22.57"N/23°04'52.01"E	1519	*Picea abies* open forest
29	29	Romania, Sud-Muntenia	45°17'18.78"N/23°41'22.64"E	1580	*Picea abies* forest
30	22	Romania, Vest	45°16'16.88"N/22°53'40.86"E	1133	*Picea abies* forest
31	23	Romania, Sud-Muntenia	45°19'36.48"N/25°26'27.15"E	1478	*Picea abies* forest

32	23	Romania, Nord-Vest	47°35'56.97"N/24°54'53.28"E	1121	*Picea abies* forest
33	23	Slovakia, Prešov Region	49°07'25.15"N/20°03'06.67"E	1372	*Larix decidua* open and burned forest
34	23	Spain, León	43°06'24.09"N/04°59'41.08"W	1364	*Fagus sylvatica* forest
35	21	Sweden, Jämtland	62°19'26.00"N/14°25'46.03"E	600	*Picea abies* forest
36	23	Sweden, Hälsingland	61°56'26.00"N/15°36'27.10"E	230	Mixed coniferous forest
37	24	Austria, Tirol	47°01'00.49"N/11°10'15.48"E	1683	*Picea abies* forest
38	25	Austria, Carinthia	47°00'50.61"N/13°24'46.95"E	1205	*Picea abies* and *Larix decidua* forest
39	21	Slovenia, Gorizia	46°23'03.26"N/13°46'57.83"E	806	*Larix decidua* sparse trees
40	21	Italy, Trento	46°13'53.20"N/10°50'54.67"E	1848	*Picea abies* forest
41	22	Italy, Lombardia	46°23'23.38"N/10°30'10.70"E	2173	*Pinus cembra* and *Larix decidua* open forest
42	21	Switzerland, Schwyz	47°06'35.08"N/08°52'58.03"E	1614	*Picea abies* isolated trees
43	25	Sweden, Örebro	59°16'43.02"N/14°52'59.53"E	197	*Picea abies* forest
44	20	Sweden, Värmland	59°23'04.06"N/13°20'47.04"E	65	Mixed forest
45	25	Sweden, Värmland	59°30'00.02"N/11°52'20.06"E	15	*Pinus sylvestris* and *Picea abies* young forest
46	24	Norway, Telemark	59°36'13.52"N/09°25'03.48"E	474	*Pinus sylvestris* and *Abies alba* open forest
47	24	Norway, Telemark	59°35'24.97"N/08°37'15.31"E	387	*Picea abies* forest
48	23	Norway, Telemark	59°27'52.90"N/08°01'18.22"E	896	Mixed forest margin near a farm
49	25	Norway, Hordaland	60°35'13.02"N/06°36'32.05"E	242	*Picea abies* forest
50	25	Norway, Sogn og Fjordane	61°16'56.05"N/07°00'05.05"E	381	*Picea abies* and *Pinus sylvestris* forest
51	12	Norway, Sogn og Fjordane	61°38'46.49"N/07°16'38.26"E	253	*Picea abies* and *Pinus sylvestris* trees in a bog
52	25	Norway, Sogn og Fjordane	61°30'07.06"N/07°47'44.05"E	842	*Betula pubescens* forest
53	25	Norway, Sør-Trøndelag	62°39'39.02"N/09°53'24.02"E	554	*Pinus sylvestris* trees near a crop
54	25	Norway, North-Trondelag	63°59'10.56"N/11°26'47.21"E	68	*Picea abies* forest
55	25	Norway, Sogn og Fjordane	61°46'30.09"N/06°29'52.02"E	588	*Pinus sylvestris* open forest
56	25	Norway, North-Trondelag	64°14'34.00"N/12°09'56.01"E	41	*Picea abies* and *Pinus sylvestris* forest
57	25	Norway, North-Trondelag	64°32'11.00"N/12°25'54.04"E	110	*Picea abies* forest
58	25	Norway, Nordland	65°06'03.08"N/13°20'35.00"E	304	*Picea abies*, *Pinus sylvestris* and *Betula pubescens* open forest
59	28	Norway, Nordland	66°28'09.05"N/15°03'09.09"E	255	*Picea abies* forest
60	25	Sweden, Västerbotten	64°42'09.00"N/16°42'30.02"E	379	*Picea abies* forest
61	25	Sweden, Västerbotten	65°39'13.04"N/15°30'32.01"E	484	*Picea abies* and *Betula pubescens* forest
62	25	Sweden, Jämtland	63°54'24.06"N/16°21'19.04"E	199	*Picea abies* forest
63	25	Sweden, Gävleborg	61°50'30.89"N/17°11'45.59"E	30	Mixed human-disturbed forest
64	25	France, Pyrenees	42°28'46.23"N/02°18'52.87"E	1795	*Pinus sylvestris* forest

Table S2. List of selected specimens for the phylogenetic analysis and GenBank accession numbers for each locus. Newly generated sequences are in bold. The first two numbers of the specimen code indicate the population membership. Morphological and chemical characters of specimens can be seen in Table S7.

Specimen code	ITS	IGS	GAPDH	FRBi13	FRBi15	FRBi16	FRBi18	FRBi19	Algal ITS
***Bryoria glabra* L198b**	KJ576730	**pendent**	KJ599483	**KY945122**	-	**pendent**	-	**KY945185**	KJ576651
L01.01	KY026912	KY026957	KY027002	**KY945137**	KY026826	KY002703	**KY945156**	**KY945186**	**KY945078**
L01.17	KY026893	KY026939	KY026986	**KY945138**	KY026800	KY002694	**KY945157**	**KY945187**	**KY945079**
L02.20	KY026929	KY026976	KY027019	**KY945123**	KY026870	KY002734	**KY945158**	**KY945188**	**KY945093**
L03.07	KY026930	KY026977	KY027020	**KY945124**	KY026871	KY002708	**KY945159**	**KY945189**	**KY945094**
L04.03	KY026917	KY026962	KY027007	**KY945125**	KY026842	-	**KY945160**	**KY945190**	**KY945095**
L05.17	KY026931	KY026978	KY027021	**KY945126**	KY026872	KY002709	**KY945161**	**KY945191**	**KY945096**
L06.05	KY026913	KY026958	KY027003	**KY945127**	KY026827	-	**KY945162**	**KY945192**	**KY945102**
L06.10	KY026894	KY026940	KY026987	**KY945128**	KY026801	KY002715	**KY945163**	**KY945193**	**KY945101**
L07.03	KY026932	KY026979	KY027022	**KY945129**	KY026873	KY002735	**KY945164**	**KY945194**	**KY945105**
L07.15	KY026895	KY026941	KY026988	**KY945130**	KY026802	KY002716	**KY945165**	**KY945195**	**KY945103**
L07.19	KY026933	KY026980	KY027023	**KY945131**	KY026874	KY002736	**KY945166**	**KY945196**	**KY945104**
L08.12	KY026896	KY026942	KY026989	**KY945132**	KY026803	KY002695	**KY945167**	**KY945197**	**KY945106**
L08.19	KY026934	KY026981	KY027024	**KY945133**	KY026875	KY002737	**KY945168**	**KY945198**	**KY945107**
L08.20	KY026935	KY026982	KY027025	**KY945134**	KY026876	-	**KY945169**	**KY945199**	**KY945108**
L09.04	KY026918	KY026963	KY027008	**KY945135**	KY026843	KY002743	**KY945170**	**KY945200**	**KY945109**
L09.07	KY026919	KY026964	KY027009	**KY945136**	KY026844	KY002744	**KY945171**	**KY945201**	**KY945110**
L10.03	KY026914	KY026959	KY026828	**KY945139**	KY026828	KY002721	**KY945172**	**KY945202**	**KY945081**
L10.13	KY026936	KY026983	KY027026	**KY945140**	KY026877	KY002722	**KY945173**	**KY945203**	**KY945080**
L11.15	KY026915	KY026960	KY027005	**KY945141**	KY026829	KY002704	**KY945174**	**KY945204**	**KY945082**
L12.03	KY026902	KY026947	KY026994	**KY945142**	KY026813	-	-	**KY945205**	**KY945084**
L12.05	KY026903	KY026948	KY026995	**KY945143**	KY026814	KY002730	**KY945175**	**KY945206**	**KY945085**
L12.11	KY026937	KY026984	KY027027	**KY945144**	KY026878	KY002738	**KY945176**	**KY945207**	**KY945083**
L13.03	KY026897	KY026943	KY026990	**-**	KY026804	KY002696	**KY945177**	**KY945208**	**KY945087**
L13.12	KY026938	KY026985	KY027028	**KY945145**	KY026879	-	**KY945178**	**KY945209**	**KY945086**
L14.02	KY026898	KY026944	KY026991	**KY945146**	KY026805	KY002717	**KY945179**	**KY945210**	**KY945088**
L15.15	KY026899	KY026945	KY026992	**KY945147**	KY026807	KY002718	**KY945180**	**KY945211**	**KY945089**
L15.21	KY026901	KY026949	KY026996	**KY945148**	KY026817	-	**KY945181**	**KY945212**	-
L16.15	KY026916	KY026961	KY027006	**KY945149**	KY026830	-	-	**KY945213**	**KY945090**
L16.17	KY026920	-	KY027010	**KY945150**	KY026845	-	**KY945182**	**KY945214**	**KY945091**
L16.21	KY026900	KY026946	KY026993	**KY945151**	KY026808	KY002719	**KY945183**	**KY945215**	**KY945092**
L54.19	**KY945112**	**KY945115**	-	**KY945152**	**KY945118**	-	-	-	**KY945097**
L54.20	**KY945113**	-	-	**KY945153**	**KY945119**	-	-	**KY945216**	**KY945098**
L58.08	**KY945114**	**KY945116**	-	**KY945154**	**KY945120**	-	-	**KY945217**	-
L58.17	**KY945111**	**KY945117**	-	**KY945155**	**KY945121**	-	**KY945184**	-	**KY945099 & KY945100**

Table S3. Primer information.

Marker	Description	Primer forward (5'–3')	Source	Primer reverse (5'–3')	Source
ITS	Internal transcribed spacers of the fungal nuclear rDNA including the 5.8S region	ITS1–F: CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns (1993)	ITS4: TCCTCCGCTTATTGATATGC	White *et al*. (1990)
IGS	Intergenic spacer of the nuclear rDNA	IGS12b: AGTCTGTGGATTAGTGGCCG	Printzen & Ekman (2002)	SSU72R: TTGCTTAAACTTAGACATG	Gargas & Taylor (1992)
GAPDH	Glyceraldehyde 3–phosphate dehydrogenase gene partial sequence	Gpd1–LM: ATTGGCCGCATCGTCTTCCGCAA	Myllys et al. (2002)	Gpd2–LM: CCACTCGTTGTCGTACCA	Myllys *et al*. (2002)
FRBi13	Flanking region of *Bryoria* sect. *Implexae* microsatellite marker 13	FRBi13f: TGATTCCACTCCCTAAACCT	This paper	FRBi13r: GTTGCCGTAGATCCCAGTATT	This paper
FRBi15	Flanking region of *Bryoria* sect. *Implexae* microsatellite marker 15	FRBi15f: GTCATAAGGGTATCAATCC	See Chapter 4	FRBi15r: TGAAAAGGTTTGGTGACTC	See Chapter 4
FRBI16	Flanking region of *Bryoria* sect. *Implexae* microsatellite marker 16	FRBi16f: CGAGGTTTCAGGAAAGGGAA	See Chapter 4	FRBi16r: AGGAAGTGATGTCGAGGT	See Chapter 4
FRBi18	Flanking region of *Bryoria* sect. *Implexae* microsatellite marker 18	FRBi18f: TCTCGATACCCTCCCTTTC	This paper	FRBi18r: ATTTCTTCTCAGCACTCTA	This paper
FRBi19	Flanking region of *Bryoria* sect. *Implexae* microsatellite marker 19	FRBi19f: TGACTTGAGTATGGTGCTGC	This paper	FRBi19r: TCTTTGCTTCTATCGCCTTCT	This paper
Algal ITS	Internal transcribed spacers of the algal nuclear rDNA including the 5.8S region	ITS1T: GGAAGGATCATTGAATCTATCGT	Kroken & Taylor (2000)	ITS4T: GGTTCGCTCGCCGCTACTA	Kroken & Taylor (2000)

Table S4. Microsatellite data matrix used for the analysis after remove unexpected allele sizes and specimens with missing data. The first two of the specimen numbers corresponds to the population code.

Specimen	Bi3	Bi4	Bi5	Bi6	Bi7	Bi8	Bi10	Bi11	Bi12	Bi13	Bi14	Bi16	Bi18	Bi 19
L01-01	281	322	139	114	123	367	434	316	106	120	361	409	391	350
L01-02	281	322	139	114	123	367	434	316	106	120	361	409	391	350
L01-03	281	322	139	114	123	367	434	316	106	120	361	409	391	350
L01-04	279	322	139	114	123	367	434	316	103	108	361	407	391	350
L01-05	279	322	139	114	123	367	434	316	103	108	361	407	391	350
L01-06	279	322	139	114	123	367	434	316	103	108	361	407	391	350
L01-07	279	322	139	114	123	367	434	316	103	108	361	407	391	350
L01-08	279	322	135	123	133	369	434	316	103	108	365	407	393	350
L01-09	279	322	135	114	133	367	434	316	103	120	361	409	393	352
L01-10	279	322	127	117	133	367	436	316	100	93	365	405	393	346
L01-11	279	322	127	117	133	367	436	316	100	93	365	405	391	346
L01-12	279	322	127	117	133	367	436	316	100	123	365	405	393	346
L01-13	279	322	127	138	133	369	436	316	100	93	365	405	393	346
L01-14	279	322	135	114	123	367	434	317	103	93	361	407	391	352
L01-15	279	322	135	114	123	367	434	317	103	93	361	407	391	352
L01-16	279	322	127	117	133	367	436	316	100	93	365	405	393	346
L01-17	279	322	127	117	133	367	436	316	100	93	365	405	393	346
L01-18	279	322	135	123	133	369	434	316	103	126	365	407	393	350
L01-19	279	322	139	135	123	369	434	316	103	108	361	407	391	350
L01-20	279	322	127	117	133	367	436	316	100	93	365	405	393	346
L02-01	279	322	139	123	123	367	434	316	103	108	361	411	393	352
L02-02	279	322	139	123	123	367	434	316	103	108	361	411	393	352
L02-03	279	322	139	123	123	367	434	316	103	108	361	411	393	352
L02-04	279	322	139	123	123	367	434	316	103	108	361	411	393	352
L02-05	279	322	139	123	123	367	434	316	103	108	361	411	393	352
L02-06	279	322	135	114	123	369	434	316	115	126	361	409	391	352
L02-07	279	322	139	114	123	367	434	316	145	108	361	409	393	352
L02-08	279	322	139	123	123	367	434	316	103	108	361	411	393	352
L02-09	279	322	135	123	123	369	434	316	118	108	361	411	393	350
L02-10	279	322	139	135	123	367	434	316	118	123	361	407	393	352
L02-11	279	322	139	114	123	367	434	316	106	114	361	413	393	352
L02-12	279	324	139	114	123	369	434	318	103	120	361	409	391	352
L02-13	279	322	135	114	123	369	434	316	115	126	361	409	391	352
L02-14	279	322	135	114	123	369	434	316	115	126	361	409	391	352
L02-15	281	322	139	114	123	367	434	316	103	93	361	409	391	352
L02-16	279	322	139	114	123	367	434	316	103	123	361	407	393	352
L02-17	279	322	135	114	123	369	434	316	115	126	361	409	391	352
L02-18	279	322	139	114	123	367	434	316	106	114	361	413	393	352
L02-19	279	322	135	135	123	369	434	316	103	108	361	407	391	350
L02-20	279	322	139	114	123	367	434	316	106	114	361	413	393	352
L03-01	279	324	139	114	123	369	434	318	103	123	361	409	393	352
L03-02	279	324	135	114	123	369	434	318	103	126	361	411	393	350
L03-03	263	322	139	135	123	367	434	316	115	129	361	411	391	350
L03-04	279	322	139	123	123	367	434	316	103	93	361	411	393	350

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L31-06	279	324	127	165	133	367	436	318	100	93	361	405	391	346
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L43-02	279	322	139	114	123	367	436	316	118	126	361	411	397	352
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L43-21	279	322	139	135	123	369	434	316	115	108	361	411	391	352
L43-22	281	322	139	135	123	367	434	316	103	108	361	411	393	350

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L43-25	279	322	135	114	123	367	434	316	100	108	361	411	391	346
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L44-16	279	322	139	114	133	367	436	316	103	108	361	411	397	352
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L45-25	279	322	139	135	123	367	434	316	103	123	361	411	393	346
L46-01	279	322	139	135	133	367	434	316	103	126	361	411	397	346
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L46-06	279	322	139	123	123	369	434	316	124	108	361	413	391	346
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L47-16	279	322	139	159	123	367	436	316	115	90	365	407	393	346
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L54-06	277	316	131	123	123	385	436	310	100	108	365	411	391	344
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L55-25	279	322	135	135	123	367	434	316	103	123	361	411	391	350
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L59-22	279	322	127	162	133	369	436	316	100	93	365	411	393	346
L59-23	279	324	127	162	133	369	436	318	100	93	365	407	393	346
L59-24	279	324	127	165	133	369	436	318	100	108	365	407	393	346
L59-25	279	324	127	165	133	369	436	318	100	108	365	407	393	346
L59-26	279	324	127	165	133	369	436	318	100	108	365	407	393	346
L59-27	279	322	139	135	123	367	434	316	124	105	361	411	397	346
L59-28	279	324	111	165	133	369	436	318	100	108	365	407	393	346
L60-01	279	322	135	135	133	369	436	316	100	108	365	407	393	346

L60-02	279	324	127	159	133	369	436	318	100	93	365	407	393	346
L60-03	279	322	139	114	123	369	434	316	115	108	361	411	391	352
L60-04	279	322	127	117	133	365	436	316	100	93	365	407	393	346
L60-05	279	322	127	117	133	369	436	316	100	93	365	407	393	346
L60-06	279	322	139	135	123	367	434	316	103	108	361	411	379	350
L60-07	279	322	127	159	133	369	436	316	100	108	365	407	393	346
L60-08	279	322	139	135	123	367	434	316	133	105	361	411	391	352
L60-09	279	322	127	162	133	369	436	316	100	93	365	407	393	346
L60-10	279	322	127	156	133	369	436	316	100	93	365	407	393	346
L60-11	279	322	127	117	133	367	436	316	100	93	365	407	393	346
L60-12	279	324	127	165	133	369	436	318	100	93	365	407	393	346
L60-13	279	322	127	162	133	369	436	316	100	93	365	407	393	346
L60-14	279	322	127	159	133	369	436	316	100	93	365	413	393	346
L60-15	279	322	139	135	123	367	434	316	103	108	361	413	379	350
L60-17	281	322	135	123	123	367	436	316	103	102	361	411	397	352
L60-18	279	322	135	123	123	367	436	316	118	108	361	411	391	350
L60-19	279	322	103	141	128	367	436	316	100	93	365	407	391	346
L60-20	279	322	139	114	123	369	434	316	103	93	361	411	393	352
L60-21	279	322	127	168	133	369	436	316	100	93	365	407	393	346
L60-22	279	322	127	168	133	369	436	316	100	93	365	407	393	346
L60-23	279	322	127	117	133	367	436	316	100	93	365	407	393	346
L60-24	279	322	139	123	123	369	434	316	103	105	361	411	391	350
L60-25	279	322	139	123	133	367	434	316	103	126	361	407	391	350
L61-01	277	316	131	132	123	385	434	310	100	93	365	407	393	344
L61-02	279	322	139	144	123	369	434	316	115	105	361	411	393	352
L61-03	279	322	127	162	133	369	436	316	100	93	365	407	393	346
L61-04	281	322	135	123	123	367	434	316	103	105	361	411	393	350
L61-05	279	322	127	117	133	365	436	316	100	93	365	407	393	346
L61-06	279	324	127	117	133	365	436	318	100	93	365	407	393	346
L61-08	281	322	139	141	123	367	434	316	103	93	361	411	397	350
L61-10	277	316	131	132	123	385	434	310	100	105	365	407	393	344
L61-11	279	324	135	123	123	367	434	318	103	93	361	413	393	352
L61-12	279	322	139	135	123	367	434	316	103	93	361	411	393	352
L61-13	277	316	131	132	133	385	434	310	100	105	365	407	393	344
L61-14	279	322	139	135	123	367	434	316	103	93	361	409	393	350
L61-15	279	324	139	123	123	369	434	318	115	93	361	409	391	352
L61-16	279	324	131	117	133	365	436	318	100	93	365	407	393	346
L61-17	277	316	131	135	133	385	436	310	100	105	365	407	393	344
L61-18	277	316	131	132	133	385	434	310	100	105	365	407	393	344
L61-19	279	322	127	117	133	365	436	316	100	93	365	407	393	346
L61-20	277	316	131	132	133	385	434	310	100	105	365	407	393	344
L61-21	279	322	127	168	133	369	436	316	100	93	365	407	393	346
L61-22	279	322	135	135	123	367	434	315	118	108	361	411	393	352
L61-24	279	322	127	168	133	369	436	316	100	93	365	407	393	346
L61-25	279	322	127	165	133	369	436	316	100	93	365	407	393	346
L62-01	279	322	127	159	133	369	436	316	100	93	365	407	393	346

L62-02	279	322	139	123	123	369	434	316	115	126	361	407	379	350
L62-03	279	322	127	165	133	369	436	316	100	93	365	407	391	346
L62-04	279	322	127	165	133	369	436	316	100	93	365	407	393	346
L62-05	279	322	127	165	133	369	436	316	100	93	365	407	391	346
L62-06	279	322	127	165	133	369	436	316	100	93	365	407	391	346
L62-07	279	322	147	123	133	367	436	316	100	129	365	407	393	346
L62-08	279	322	127	117	133	367	436	316	100	93	365	407	397	346
L62-09	279	322	127	159	133	367	436	316	100	93	365	407	393	346
L62-10	279	322	127	159	133	369	436	316	100	93	365	407	393	346
L62-11	279	322	127	117	133	367	436	316	100	93	365	407	397	346
L62-12	279	322	127	117	133	367	436	316	100	93	365	407	397	346
L62-13	279	322	127	165	133	369	436	316	100	93	365	407	397	346
L62-14	279	322	139	135	123	367	434	316	103	123	361	411	393	346
L62-15	279	322	127	159	133	369	436	316	100	93	365	407	393	346
L62-16	279	322	127	117	133	367	436	316	100	93	365	407	397	346
L62-17	279	322	127	117	133	367	436	316	100	93	365	407	397	346
L62-18	279	324	139	135	123	369	434	318	100	93	361	409	391	350
L62-19	279	322	127	165	133	369	436	316	100	93	365	407	393	346
L62-20	279	322	127	168	133	369	436	316	100	93	365	407	393	346
L62-21	279	324	127	165	133	369	436	318	100	93	365	407	393	346
L62-22	279	322	127	168	133	369	436	316	100	93	365	407	393	346
L62-23	279	322	127	159	133	367	436	316	100	93	365	407	393	346
L62-24	279	322	127	117	133	367	436	316	100	93	365	407	393	346
L63-01	279	322	139	123	123	369	434	316	124	108	361	411	391	346
L63-02	279	322	139	123	123	367	434	316	115	123	361	411	393	346
L63-03	279	322	135	135	123	367	434	316	118	93	361	411	397	352
L63-04	279	322	139	135	123	367	434	316	103	126	361	411	397	352
L63-05	279	322	139	135	123	367	434	316	115	126	361	411	397	346
L63-06	279	322	139	141	133	369	434	316	115	108	361	411	397	352
L63-07	281	322	139	135	123	367	434	316	115	126	361	411	397	346
L63-08	279	322	139	135	123	367	434	316	124	108	361	411	393	346
L63-09	279	322	139	123	123	369	434	316	103	93	361	409	391	350
L63-10	281	322	139	135	123	367	434	316	115	108	361	411	397	350
L63-11	279	322	139	135	123	367	434	316	115	126	361	411	397	346
L63-12	279	322	139	123	123	367	434	316	115	123	361	411	393	346
L63-13	279	322	139	135	123	367	436	316	115	123	361	411	391	346
L63-14	279	322	139	123	123	369	434	316	124	108	361	411	391	346
L63-15	279	322	139	135	123	367	434	316	103	108	361	411	391	346
L63-16	279	322	139	135	123	367	434	316	118	108	361	411	397	346
L63-17	281	324	139	135	123	367	434	318	100	105	361	411	391	344
L63-18	279	322	139	135	123	367	434	316	133	126	361	411	397	350
L63-19	281	322	139	135	123	367	434	316	115	108	361	411	397	346
L63-20	279	322	139	141	123	367	434	316	103	108	361	411	391	346
L63-21	279	322	139	123	133	369	434	316	115	126	361	411	397	346
L63-22	279	322	139	135	123	367	434	316	115	132	361	413	397	346
L63-23	279	322	135	135	123	367	434	316	115	126	361	413	397	346

L63-24	279	322	139	135	123	367	434	316	121	123	361	411	393	346
L63-25	279	322	139	123	123	369	434	316	124	108	361	413	391	346
L64-01	279	322	127	138	133	369	436	316	100	93	365	407	393	346
L64-02	279	322	127	117	133	369	436	316	100	93	365	407	393	346
L64-03	279	322	127	165	133	369	436	316	100	93	365	407	393	346
L64-04	279	324	127	135	133	369	434	318	100	93	365	407	397	346
L64-05	279	322	127	114	133	367	436	316	100	93	365	407	397	346
L64-06	279	322	127	159	133	369	436	316	100	93	365	407	393	346
L64-07	279	322	127	114	133	367	436	316	100	93	365	407	397	346
L64-08	279	322	127	165	133	369	436	316	100	93	365	407	393	346
L64-09	279	322	127	117	133	369	436	316	100	93	365	407	393	346
L64-10	281	322	127	117	133	367	436	316	100	93	365	407	393	346
L64-11	281	322	127	117	133	367	436	316	100	93	365	407	393	346
L64-12	279	324	127	117	133	369	436	318	100	93	365	407	393	346
L64-13	281	322	127	159	133	367	436	316	100	108	365	407	393	346
L64-14	281	322	127	159	133	367	436	316	100	108	365	407	393	346
L64-15	281	322	127	117	133	367	436	316	100	93	365	407	393	346
L64-16	279	322	127	114	133	367	436	316	100	93	365	407	393	346
L64-17	279	322	127	114	133	367	436	316	100	93	365	407	393	346
L64-18	279	322	127	114	133	367	436	316	100	93	365	407	393	346
L64-19	281	322	127	159	133	367	436	316	100	108	365	407	393	346
L64-20	281	322	127	159	133	367	436	316	100	108	365	407	393	346
L64-21	279	322	127	117	133	367	436	316	100	93	365	407	393	346
L64-22	279	322	127	114	133	369	436	316	100	93	365	407	393	346
L64-23	279	326	127	165	133	369	436	320	100	93	365	407	393	346
L64-24	279	322	127	114	133	367	436	316	100	93	365	407	393	346
L64-25	279	322	127	117	133	369	436	316	100	93	365	407	391	346

Table S5. Allelic richness (AR) and private allelic richness (PAR) detected in each type of substrate. Standard deviations are showed in brackets.

	Twigs			Branches			Trunks			Rock	
	AR	**PAR**		**AR**	**PAR**		**AR**	**PAR**		**AR**	**PAR**
n:203	5.93 (1.15)	0.57 (0.21)	n: 30	3.57 (0.63)	0.07 (0.07)	n:256	6.07 (1.15)	0.68 (0.29)	n: 37	2.79 (0.39)	0.00 (0.00)
AR/n	0.029	-	AR/n	0.119	-	AR/n	0.024	-	AR/n	0.075	-

Table S6. Global Analysis of Molecular Variance (AMOVA) using 14 loci, 1359 individuals, 64 populations and the 7 geographical regions from Fig. 1. F_{SC} = 0.21381, F_{ST} = 0.23187 and F_{CT} = 0.02297, statistically significant with $P \leq 0.035$.

Source of variation	df	Sum of squares	Variance components	% of variation
Among regions	6	211.80	0.089	2.30
Among populations within regions	57	1146.23	0.808	20.89
Within populations	1295	3846.72	2.970	76.81
Total	1358	5204.75	3.867	

Table S7. Morphological and chemical data of *Bryoria* taxa studied.

code	Cluster	species	chemotype	colour	soralia	colour	base	pseudocifellae	angles	apothecia	parasites
L01-01	2	fuscescens	PSO	dark brown	fissural and tuberculated	dark	paler	conspicous		absent	absent
L01-02	2	fuscescens	PSO	dark brown	fissural and tuberculated	dark	paler	conspicous		absent	absent
L01-03	2	fuscescens	PSO	dark brown	absent	dark	paler	conspicous		absent	absent
L01-04	2	fuscescens	PSO	dark brown	absent	dark	normal	conspicous		absent	absent
L01-05	2	fuscescens	PSO	dark brown	fissural and tuberculated	dark	normal	inconspicous		absent	absent
L01-06	2	fuscescens	PSO	dark gray	fissural and tuberculated	dark	normal	inconspicous		absent	absent
L01-07	2	fuscescens	PSO	dark brown	fissural and tuberculated	dark	normal	inconspicous		absent	absent
L01-08	2	fuscescens	NOR	dark brown	fissural and tuberculated	dark	normal	inconspicous		absent	absent
L01-09	2	fuscescens	NOR	dark brown	fissural	dark	normal	inconspicous		absent	absent
L01-10	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous		absent	absent
L01-11	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous		absent	absent
L01-12	1	capillaris	BAR, ALE	brown	absent	medium	paler	inconspicous		absent	absent
L01-13	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous		absent	absent
L01-14	2	fuscescens	NOR	dark brown	fissural and tuberculated	dark	normal	inconspicous		absent	absent
L01-15	2	fuscescens	NOR	black	fissural	dark	normal	inconspicous		absent	absent
L01-16	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous		absent	absent
L01-17	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous		absent	absent
L01-18	2	fuscescens	NOR	black	absent	dark	normal	inconspicous		absent	absent
L01-19	2	fuscescens	NOR	black	fissural	dark	normal	inconspicous		absent	absent
L01-20	1	sp	ABS	pale gray	absent	pale	normal	inconspicous		absent	absent
L02-01	2	fuscescens	FUM	dark brown	tuberculated	dark	normal	inconspicous		absent	absent
L02-02	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous		absent	absent
L02-03	2	fuscescens	FUM	black	fissural and tuberculated	dark	normal	inconspicous		absent	absent
L02-04	2	fuscescens	FUM	black	fissural and tuberculated	dark	normal	inconspicous		absent	absent
L02-05	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous		absent	absent
L02-06	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous		absent	absent
L02-07	2	fuscescens	FUM	black	fissural	dark	normal	inconspicous		absent	absent
L02-08	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous		absent	absent
L02-09	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	paler	inconspicous		absent	absent
L02-10	2	fuscescens	FUM	dark gray	fissural	dark	paler	inconspicous		absent	absent
L02-11	2	fuscescens	NOR	black	tuberculated	dark	normal	inconspicous		absent	absent

L02-12	2	fuscescens	FUM	black	fissural and tuberculated	dark	paler	inconspicous		absent	absent
L02-13	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous		absent	present
L02-14	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	paler	inconspicous		absent	present
L02-15	2	fuscescens	NOR	black	fissural and tuberculated	dark	normal	inconspicous		absent	present
L02-16	2	fuscescens	NOR	dark brown	fissural and tuberculated	dark	paler	inconspicous		absent	present
L02-17	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	paler	inconspicous		absent	present
L02-18	2	fuscescens	NOR	dark brown	fissural and tuberculated	dark	paler	inconspicous		absent	present
L02-19	2	fuscescens	NOR	black	fissural and tuberculated	dark	paler	inconspicous		absent	present
L02-20	2	fuscescens	FUM	black	fissural and tuberculated	dark	paler	inconspicous		absent	present
L03-01	2	sp	FUM	brown	tuberculated	medium	normal	inconspicous	obtuse	absent	absent
L03-02	2	sp	FUM	brown	tuberculated	medium	normal	inconspicous	obtuse	absent	absent
L03-03	2	sp	UNK	gray	tuberculated	medium	normal	inconspicous	obtuse	absent	absent
L03-04	2	sp	UNK	brown	tuberculated	medium	normal	inconspicous	obtuse	absent	absent
L03-05	2	sp	FUM	brown	tuberculated	medium	normal	inconspicous	obtuse	absent	absent
L03-06	2	sp	NO SUBS	brown	tuberculated	medium	paler	inconspicous	obtuse	absent	absent
L03-07	2	sp	FUM	gray	tuberculated	medium	paler	inconspicous	obtuse	absent	absent
L03-08	1	sp	FUM	brown	absent	medium	normal	inconspicous	obtuse	absent	absent
L03-09	2	sp	FUM	brown	tuberculated	medium	normal	inconspicous	obtuse	absent	absent
L03-10	1	sp	FUM	brown	absent	medium	normal	inconspicous	obtuse	absent	present
L03-11	2	sp	FUM	pale brown	tuberculated	pale	paler	inconspicous	obtuse	absent	absent
L03-12	2	sp	FUM	brown	tuberculated	medium	normal	inconspicous	mixed	absent	present
L03-13	1	sp	PSO	white	absent	pale	normal	inconspicous	obtuse	absent	absent
L03-14	1	sp	PSO	pale gray	absent	pale	normal	inconspicous	obtuse	absent	absent
L03-15	2	sp	FUM	dark brown	tuberculated	dark	normal	inconspicous	acute	absent	absent
L03-16	2	sp	FUM	dark brown	fissural and tuberculated	dark	paler	inconspicous	obtuse	absent	present
L03-17	2	sp	FUM	dark brown	tuberculated	dark	normal	inconspicous	obtuse	absent	absent
L03-18	2	sp	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous	obtuse	absent	absent
L03-19	2	sp	FUM	dark brown	tuberculated	dark	normal	inconspicous	mixed	absent	absent
L03-20	2	sp	NO SUBS	brown	absent	medium	normal	inconspicous	obtuse	absent	absent
L04-01	2	fuscescens	NOR	greenish-black	tuberculated	dark	normal	inconspicous	acute	absent	absent
L04-02	2	fuscescens	FUM	greenish-black	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L04-03	2	fuscescens	NOR	greenish-black	absent	dark	normal	inconspicous	acute	absent	absent
L04-04	2	fuscescens	NOR	greenish-black	tuberculated	dark	normal	inconspicous	acute	absent	absent
L04-05	2	fuscescens	NOR	greenish-black	tuberculated	dark	normal	inconspicous	acute	absent	absent
L04-06	2	fuscescens	NOR	greenish-black	tuberculated	dark	normal	inconspicous	acute	absent	absent
L04-07	2	fuscescens	NOR	dark gray	tuberculated	dark	normal	inconspicous	acute	absent	absent
L04-08	2	fuscescens	NOR	greenish-black	tuberculated	dark	normal	inconspicous	acute	absent	absent

L04-09	2	fuscescens	FUM	gray	fissural and tuberculated	medium	normal	inconspicous	acute	absent	absent
L04-10	2	fuscescens	FUM	brown	fissural and tuberculated	medium	normal	inconspicous	acute	absent	absent
L04-11	2	fuscescens	FUM	grayish-brown	fissural and tuberculated	dark	paler	inconspicous	acute	absent	absent
L04-12	2	fuscescens	NOR	dark gray	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L04-13	2	fuscescens	NOR	dark green	tuberculated	dark	paler	inconspicous	acute	absent	absent
L04-14	2	fuscescens	NOR	greenish-gray	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L04-15	2	fuscescens	FUM	dark green	fissural and tuberculated	dark	paler	inconspicous	acute	absent	absent
L05-01	2	fuscescens	FUM	brown	tuberculated	medium	normal	inconspicous	acute	absent	absent
L05-02	2	fuscescens	FUM	brown	tuberculated	medium	normal	inconspicous	acute	absent	absent
L05-03	2	fuscescens	FUM	dark brown	tuberculated	dark	normal	inconspicous	acute	absent	absent
L05-04	2	fuscescens	FUM	dark brown	tuberculated	dark	normal	inconspicous	acute	absent	absent
L05-05	2	fuscescens	FUM	brown	fissural and tuberculated	medium	normal	inconspicous	acute	absent	absent
L05-06	2	fuscescens	FUM	dark olive	absent	dark	normal	inconspicous	acute	absent	absent
L05-07	2	fuscescens	FUM	dark olive	tuberculated	dark	normal	inconspicous	acute	absent	absent
L05-08	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L05-09	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L05-10	2	fuscescens	FUM	brown	fissural and tuberculated	medium	normal	inconspicous	acute	absent	absent
L05-11	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L05-12	2	fuscescens	FUM	brown	absent	medium	normal	inconspicous	acute	absent	absent
L05-13	2	fuscescens	FUM	dark olive	tuberculated	dark	normal	inconspicous	acute	absent	absent
L05-14	2	fuscescens	FUM	brown	absent	medium	normal	inconspicous	acute	absent	absent
L05-15	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L05-16	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L05-17	2	fuscescens	FUM	brown	tuberculated	medium	normal	inconspicous	obtuse	absent	absent
L05-18	2	fuscescens	FUM	brown	fissural and tuberculated	medium	normal	inconspicous	acute	absent	absent
L05-19	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L05-20	2	fuscescens	FUM	dark brown	tuberculated	dark	normal	inconspicous	acute	absent	absent
L05-21	2	fuscescens	FUM	brown	tuberculated	medium	normal	inconspicous	mixed	absent	absent
L05-22	2	fuscescens	FUM	pale brown	fissural and tuberculated	pale	normal	inconspicous	acute	absent	absent
L06-01	2	sp	FUM	grey	tuberculated	medium	normal	conspicous	acute	absent	present
L06-02	2	implexa	FUM	brown	tuberculated	medium	normal	conspicous	obtuse	absent	present
L06-03	2	implexa	NO SUBS	dark olive	absent	dark	normal	conspicous	obtuse	absent	present
L06-04	2	sp	FUM	grey	absent	medium	normal	conspicous	acute	absent	present
L06-05	2	fuscescens	FUM, PSO	brown	tuberculated	medium	normal	inconspicous	acute	absent	present
L06-06	2	sp	NO SUBS	grey	absent	medium	normal	conspicous	obtuse	absent	present
L06-07	1	fuscescens	BAR	dark grey	absent	dark	paler	conspicous	acute	absent	present
L06-08	2	implexa	NO SUBS	dark brown	tuberculated	dark	normal	conspicous	obtuse	absent	present
L06-09	1	capillaris	BAR, ALE	pale grey	absent	pale	normal	conspicous	acute	absent	absent
L06-10	1	capillaris	BAR, ALE	olive	absent	dark	paler	conspicous	acute	absent	present

L06-11	2	fuscescens	FUM	brown	fissural and tuberculated	medium	normal	inconspicous	mixed	absent	present
L06-12	2	fuscescens	FUM	brown	absent	medium	normal	inconspicous	acute	absent	present
L06-13	2	sp	NO SUBS	brown	tuberculated	medium	normal	inconspicous	obtuse	absent	absent
L06-14	2	fuscescens	FUM	brown	fissural and tuberculated	medium	normal	inconspicous	acute	absent	absent
L06-15	2	sp	FUM	brown	fissural	medium	normal	inconspicous	obtuse	absent	absent
L06-16	2	implexa	NO SUBS	brown	absent	medium	normal	conspicous	obtuse	absent	absent
L06-17	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous	obtuse	absent	absent
L06-18	2	fuscescens	NO SUBS	dark brown	tuberculated	dark	normal	inconspicous	obtuse	absent	absent
L06-19	1	capillaris	BAR, ALE	brown	fissural and tuberculated	medium	normal	inconspicous	mixed	absent	present
L06-20	2	fuscescens	FUM	brown	fissural and tuberculated	medium	normal	inconspicous	obtuse	absent	present
L06-21	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	paler	inconspicous	mixed	absent	present
L07-01	1	capillaris	BAR, ALE	white	absent	pale	normal	inconspicous	mixed	absent	absent
L07-02	1	capillaris	BAR, ALE	white	absent	pale	normal	inconspicous	acute	absent	absent
L07-03	2	sp	FUM	dark olive	fissural and tuberculated	dark	normal	conspicous	mixed	absent	absent
L07-04	2	fuscescens	FUM	olive-brown	absent	dark	normal	inconspicous	mixed	absent	absent
L07-05	2	capillaris	BAR, ALE	dark gray	absent	dark	normal	inconspicous	mixed	absent	absent
L07-06	2	fuscescens	FUM	dark olive	fissural	dark	normal	inconspicous	mixed	absent	absent
L07-07	2	fuscescens	FUM	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L07-08	2	fuscescens	NO SUBS	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L07-09	2	fuscescens	FUM	dark olive	absent	dark	paler	inconspicous	mixed	absent	absent
L07-10	2	sp	FUM	dark olive	absent	dark	normal	conspicous	mixed	absent	absent
L07-11	2	fuscescens	NO SUBS	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L07-12	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	paler	inconspicous	acute	absent	absent
L07-13	2	fuscescens	FUM	dark gray	absent	dark	normal	inconspicous	mixed	absent	absent
L07-14	1	capillaris	BAR, ALE	pale gray	absent	pale	darker	inconspicous	acute	absent	absent
L07-15	1	capillaris	BAR, ALE	white	absent	pale	normal	inconspicous	mixed	absent	absent
L07-16	2	fuscescens	FUM	dark gray	absent	dark	normal	inconspicous	acute	absent	absent
L07-17	2	fuscescens	FUM	dark gray	absent	dark	normal	inconspicous	acute	absent	absent
L07-18	2	fuscescens	FUM	dark gray	absent	dark	normal	inconspicous	acute	absent	absent
L07-19	2	fuscescens	NO SUBS	dark gray	absent	dark	normal	inconspicous	obtuse	absent	absent
L07-20	2	fuscescens	FUM	brown	absent	medium	normal	inconspicous	mixed	absent	absent
L07-21	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L07-22	1	capillaris	BAR, ALE	gray	absent	medium	normal	inconspicous	mixed	absent	absent
L08-01	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	mixed	absent	absent
L08-02	1	capillaris	BAR, ALE	white	absent	pale	normal	inconspicous	obtuse	absent	absent
L08-03	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	mixed	absent	absent
L08-04	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	mixed	absent	absent
L08-05	1	capillaris	BAR, ALE	gray	absent	medium	normal	inconspicous	obtuse	absent	absent
L08-06	1	capillaris	BAR, ALE	white	absent	pale	normal	inconspicous	acute	absent	absent
L08-07	1	capillaris	BAR, ALE	white	absent	pale	normal	inconspicous	acute	absent	present
L08-08	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous	obtuse	absent	absent

L08-09	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	mixed	absent	present
L08-10	2	fuscescens	FUM	brown	fissural and tuberculated	medium	normal	inconspicous	acute	absent	absent
L08-11	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	mixed	absent	present
L08-12	1	capillaris	BAR, ALE	pale brown	absent	pale	normal	inconspicous	acute	absent	absent
L08-13	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	mixed	absent	present
L08-14	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	obtuse	absent	absent
L08-15	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	mixed	absent	absent
L08-16	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	mixed	absent	absent
L08-17	1	capillaris	BAR, ALE	white	absent	pale	normal	inconspicous	mixed	absent	present
L08-18	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	mixed	absent	absent
L08-19	2	fuscescens	NO SUBS	brown	fissural and tuberculated	medium	normal	inconspicous	obtuse	present	absent
L08-20	2	fuscescens	FUM	brown	tuberculated with isidiomorphs	medium	normal	inconspicous	obtuse	absent	absent
L08-21	2	fuscescens	NO SUBS	brown	tuberculated	medium	normal	inconspicous	obtuse	absent	absent
L08-22	2	fuscescens	FUM	grayish brown	tuberculated	medium	paler	inconspicous	obtuse	absent	absent
L09-01	1	fuscescens	NOR	dark olive	absent	dark	paler	inconspicous	mixed	absent	absent
L09-02	1	fuscescens	NOR	dark olive	absent	dark	paler	inconspicous	mixed	absent	absent
L09-03	1	fuscescens	NOR	dark olive	absent	dark	paler	inconspicous	mixed	absent	absent
L09-04	1	fuscescens	NOR	black	absent	dark	normal	conspicous	mixed	absent	absent
L09-05	1	fuscescens	NOR	dark olive	absent	dark	paler	inconspicous	mixed	absent	absent
L09-06	1	fuscescens	NOR	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L09-07	1	fuscescens	NOR	gray	absent	medium	normal	inconspicous	acute	absent	absent
L09-08	1	fuscescens	NOR	dark gray	absent	dark	normal	inconspicous	acute	absent	absent
L09-09	1	fuscescens	NOR	dark gray	absent	dark	paler	inconspicous	acute	absent	absent
L09-10	1	fuscescens	NOR	dark olive	absent	dark	normal	inconspicous	acute	absent	absent
L09-11	1	fuscescens	NOR	dark olive	absent	dark	normal	inconspicous	acute	absent	absent
L09-12	1	sp	NOR	dark olive	absent	dark	normal	conspicous	acute	absent	absent
L09-13	1	fuscescens	NO SUBS	dark gray	absent	dark	normal	inconspicous	obtuse	absent	absent
L09-14	1	sp	NOR	dark olive	absent	dark	normal	conspicous	mixed	absent	absent
L09-15	1	fuscescens	NOR	dark olive	absent	dark	paler	inconspicous	mixed	absent	absent
L09-16	1	sp	NOR	dark brown	absent	dark	normal	conspicous	mixed	absent	absent
L09-17	1	fuscescens	NOR	dark olive	absent	dark	paler	inconspicous	obtuse	absent	absent
L09-18	1	sp	NOR	dark brown	absent	dark	paler	conspicous	acute	absent	absent
L09-19	1	implexa	NOR	dark olive	absent	dark	normal	conspicous	obtuse	absent	absent
L09-20	1	fuscescens	NOR	dark olive	absent	dark	normal	inconspicous	mixed	absent	absent
L09-21	1	fuscescens	NOR	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L09-22	1	fuscescens	NOR	dark gray	absent	dark	paler	inconspicous	obtuse	absent	absent
L10-01	2	fuscescens	NO SUBS	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L10-02	2	fuscescens	NO SUBS	dark olive	absent	dark	normal	inconspicous	mixed	absent	absent
L10-03	1	implexa	PSO	dark olive	absent	dark	normal	conspicous	obtuse	absent	absent
L10-04	2	implexa	NO SUBS	dark olive	absent	dark	normal	conspicous	obtuse	absent	absent
L10-05	2	sp	NO SUBS	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L10-06	1	sp	PSO	brownish gray	absent	medium	normal	inconspicous	obtuse	absent	absent
L10-07	1	sp	PSO	gray	absent	medium	normal	inconspicous	obtuse	absent	absent

L10-08	1	sp	PSO	dark gray	absent	dark	normal	inconspicous	obtuse	absent	absent
L10-09	1	sp	NO SUBS	dark gray	absent	dark	normal	inconspicous	acute	absent	absent
L10-10	2	fuscescens	NO SUBS	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L10-11	1	fuscescens	NO SUBS	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L10-12	1	fuscescens	PSO	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L10-13	1	sp	NO SUBS	gray	absent	medium	normal	inconspicous	obtuse	absent	absent
L10-14	2	fuscescens	NO SUBS	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L10-15	1	sp	PSO	gray	absent	medium	normal	inconspicous	mixed	absent	absent
L10-16	1	sp	NO SUBS	dark gray	absent	dark	paler	inconspicous	acute	absent	absent
L10-18	2	fuscescens	NO SUBS	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L10-19	2	fuscescens	NO SUBS	dark olive	absent	dark	normal	inconspicous	obtuse	absent	absent
L10-20	2	fuscescens	NO SUBS	dark olive	absent	dark	normal	inconspicous	mixed	absent	absent
L10-21	1	sp	NO SUBS	gray	absent	medium	normal	inconspicous	mixed	absent	absent
L10-22	2	fuscescens	NO SUBS	near black	absent	dark	normal	inconspicous	obtuse	absent	absent
L11-01	2	fuscescens	NO SUBS	dark olive	absent	dark	paler	inconspicous	obtuse	absent	absent
L11-02	2	fuscescens	NO SUBS	dark olive	absent	dark	normal	inconspicous	mixed	absent	absent
L11-03	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous	mixed	absent	absent
L11-04	2	fuscescens	FUM	dark olive	fissural	dark	normal	inconspicous	obtuse	absent	absent
L11-05	2	fuscescens	FUM	dark olive	absent	dark	normal	conspicous	mixed	absent	absent
L11-06	2	fuscescens	PSO	dark brown	fissural and tuberculated with isidiomorphs	dark	normal	conspicous	mixed	absent	absent
L11-07	2	fuscescens	PSO	dark brown	fissural	dark	paler	inconspicous	mixed	absent	absent
L11-08	2	fuscescens	NO SUBS	brown	absent	medium	normal	inconspicous	mixed	absent	absent
L11-09	2	fuscescens	FUM	dark olive	fissural	dark	normal	inconspicous	mixed	absent	absent
L11-10	2	implexa	FUM	dark olive	fissural	dark	normal	conspicous	obtuse	absent	absent
L11-11	2	implexa	NOR	brown	absent	medium	paler	conspicous	obtuse	absent	absent
L11-12	2	fuscescens	PSO	dark olive	absent	dark	paler	inconspicous	mixed	absent	absent
L11-13	2	fuscescens	NO SUBS	dark olive	fissural and tuberculated	dark	normal	inconspicous	obtuse	absent	absent
L11-14	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous	mixed	absent	absent
L11-15	2	fuscescens	PSO	dark olive	tuberculated with isidiomorphs	dark	paler	inconspicous	obtuse	absent	absent
L11-16	2	implexa	PSO	dark olive	absent	dark	normal	conspicous	obtuse	absent	absent
L11-17	2	fuscescens	FUM	dark brown	absent	dark	normal	inconspicous	obtuse	absent	absent
L11-18	2	fuscescens	FUM	near black	fissural and tuberculated	dark	paler	inconspicous	obtuse	absent	absent
L11-19	2	fuscescens	FUM	dark brown	fissural	dark	normal	inconspicous	mixed	absent	absent
L11-20	2	fuscescens	FUM	near black	fissural and tuberculated	dark	normal	inconspicous	mixed	absent	absent
L11-21	2	fuscescens	FUM	dark brown	fissural	dark	paler	inconspicous	mixed	absent	absent
L11-22	2	fuscescens	FUM	dark brown	absent	dark	paler	inconspicous	mixed	absent	absent
L12-01	2	sp	FUM	brown	fissural and tuberculated	medium	normal	conspicous	mixed	present	absent
L12-02	2	fuscescens	FUM	brown	fissural and tuberculated	medium	normal	inconspicous	mixed	absent	absent
L12-03	2	sp	FUM	pale gray	fissural and tuberculated	pale	normal	inconspicous	mixed	absent	absent

L12-04	2	sp	FUM	pale gray	fissural and tuberculated	pale	normal	inconspicous	mixed	absent	absent
L12-05	2	fuscescens	FUM	brown	fissural and tuberculated	medium	paler	inconspicous	obtuse	present	absent
L12-07	2	sp	FUM, PSO	pale gray	fissural and tuberculated	pale	normal	inconspicous	obtuse	absent	absent
L12-08	2	sp	FUM	pale brown	fissural and tuberculated	pale	normal	inconspicous	obtuse	absent	absent
L12-09	2	sp	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous	obtuse	absent	absent
L12-10	2	fuscescens	FUM	dark brown	fissural	dark	normal	inconspicous	mixed	absent	absent
L12-11	2	sp	FUM, GYR	dark brown	fissural and tuberculated	dark	normal	inconspicous	obtuse	absent	absent
L12-12	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous	obtuse	absent	absent
L12-13	2	fuscescens	FUM	dark brown	fissural and tuberculated	dark	normal	inconspicous	obtuse	absent	absent
L12-14	2	sp	FUM	dark brown	fissural and tuberculated	dark	paler	conspicous	mixed	absent	absent
L12-15	2	fuscescens	PSO	brown	fissural	medium	normal	inconspicous	acute	absent	absent
L12-16	2	sp	GYR	gray	fissural and tuberculated	medium	normal	inconspicous	mixed	absent	absent
L12-17	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	normal	inconspicous	obtuse	absent	absent
L12-19	2	sp	FUM	greenish white	tuberculated	pale	normal	inconspicous	obtuse	absent	absent
L12-20	2	sp	FUM, GYR	pale gray	fissural and tuberculated	pale	normal	inconspicous	obtuse	absent	absent
L12-21	2	sp	FUM, PSO	pale gray	fissural and tuberculated	pale	normal	inconspicous	mixed	absent	absent
L12-22	2	capillaris	FUM, BAR, ALE	greenish white	fissural and tuberculated	pale	normal	inconspicous	mixed	absent	absent
L12-23	2	sp	FUM	brown	tuberculated	medium	normal	inconspicous	obtuse	absent	absent
L13-01	2	implexa	FUM, PSO	brown	tuberculated	medium	normal	conspicous	obtuse	present	absent
L13-02	2	capillaris	FUM, BAR, ALE	near white	tuberculated	pale	normal	inconspicous	mixed	absent	absent
L13-03	2	capillaris	FUM, BAR, ALE, PSO	near white	tuberculated	pale	normal	inconspicous	mixed	present	absent
L13-04	2	sp	NO SUBS	brown	absent	medium	normal	inconspicous	obtuse	absent	absent
L13-05	2	implexa	PSO	brown	tuberculated	medium	normal	conspicous	obtuse	present	absent
L13-06	2	capillaris	BAR, ALE	near white	absent	pale	normal	inconspicous	mixed	absent	absent
L13-07	2	fuscescens	FUM	brown	tuberculated	medium	normal	inconspicous	acute	absent	absent
L13-08	2	sp	GYR	brown	tuberculated	medium	normal	conspicous	mixed	absent	absent
L13-09	2	capillaris	FUM, BAR, ALE	near white	absent	pale	normal	inconspicous	obtuse	absent	absent
L13-10	2	sp	FUM	brown	fissural and tuberculated	medium	normal	inconspicous	obtuse	absent	absent
L13-12	2	sp	GYR	brown	absent	medium	normal	conspicous	mixed	present	absent
L13-13	2	sp	FUM	brown	fissural and tuberculated	medium	normal	inconspicous	mixed	absent	absent
L13-14	2	implexa	FUM	dark brown	tuberculated	dark	normal	conspicous	obtuse	absent	absent
L14-01	2	fuscescens	FUM, PSO	dark brown	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L14-02	1	capillaris	FUM, BAR, ALE, PSO	yellowish brown	absent	pale	normal	conspicous	mixed	absent	absent
L14-03	2	fuscescens	FUM	dark olive	tuberculated and fissural	dark	paler	inconspicous	acute	absent	absent

L14-04	2	fuscescens	FUM	dark olive	fissural	dark	paler	inconspicous	acute	absent	absent
L14-05	1	capillaris	BAR, ALE, PSO	yellowish brown	absent	pale	normal	inconspicous	acute	absent	absent
L14-06	1	capillaris	BAR, ALE, PSO	yellowish brown	absent	pale	normal	inconspicous	acute	absent	absent
L14-07	1	capillaris	BAR, ALE, PSO	yellowish brown	absent	pale	normal	inconspicous	acute	absent	absent
L14-08	1	capillaris	BAR, ALE, PSO	yellowish gray	absent	pale	normal	inconspicous	acute	absent	absent
L14-09	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	paler	inconspicous	acute	absent	absent
L14-10	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L14-11	1	capillaris	BAR, ALE, PSO	yellowish gray	absent	pale	normal	inconspicous	acute	absent	absent
L14-12	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	paler	inconspicous	acute	absent	absent
L14-13	1	capillaris	BAR, ALE, PSO	yellowish brown	absent	pale	normal	inconspicous	mixed	absent	absent
L14-15	1	capillaris	BAR, ALE, PSO	pale gray	absent	pale	normal	inconspicous	mixed	absent	absent
L14-16	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	paler	inconspicous	mixed	absent	absent
L14-17	1	capillaris	BAR, ALE, PSO	pale gray	absent	pale	normal	inconspicous	mixed	absent	absent
L14-19	1	capillaris	BAR, ALE, PSO	yellowish brown	absent	pale	normal	inconspicous	acute	absent	absent
L14-21	1	capillaris	BAR, ALE, PSO	yellowish brown	absent	pale	normal	inconspicous	acute	absent	absent
L14-22	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	paler	inconspicous	mixed	absent	absent
L15-01	2	fuscescens	FUM	dark olive	tuberculated	dark	normal	inconspicous	acute	absent	present
L15-02	2	fuscescens	FUM	dark olive	tuberculated	dark	normal	inconspicous	mixed	absent	absent
L15-03	2	fuscescens	FUM	dark olive	tuberculated	dark	paler	inconspicous	acute	absent	present
L15-04	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	paler	inconspicous	acute	absent	present
L15-05	2	fuscescens	FUM	dark olive	tuberculated	dark	normal	inconspicous	acute	absent	present
L15-06	2	fuscescens	FUM	dark olive	tuberculated	dark	paler	inconspicous	obtuse	absent	absent
L15-07	2	implexa	FUM	dark brown	tuberculated	dark	normal	conspicous	obtuse	absent	absent
L15-08	2	sp	FUM	dark olive	tuberculated	dark	normal	conspicous	acute	absent	absent
L15-09	2	fuscescens	FUM	dark olive	tuberculated	dark	normal	inconspicous	acute	absent	present
L15-10	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	normal	inconspicous	acute	absent	absent
L15-11	2	fuscescens	FUM	dark olive	tuberculated	dark	normal	inconspicous	acute	absent	absent
L15-12	2	fuscescens	FUM	dark olive	tuberculated	dark	paler	inconspicous	acute	absent	absent
L15-13	2	fuscescens	FUM	dark olive	tuberculated	dark	normal	inconspicous	acute	absent	absent
L15-14	2	fuscescens	FUM	dark olive	tuberculated	dark	normal	inconspicous	acute	absent	absent
L15-15	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	acute	absent	present
L15-16	2	sp	FUM	dark olive	tuberculated	dark	paler	conspicous	acute	absent	absent
L15-17	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	normal	inconspicous	mixed	absent	absent
L15-18	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	acute	absent	absent
L15-19	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	acute	absent	present
L15-20	2	implexa	FUM	dark olive	fissural and tuberculated	dark	paler	conspicous	obtuse	absent	present
L15-21	2	fuscescens	FUM	dark olive	fissural and tuberculated	dark	normal	inconspicous	mixed	absent	present

L15-22	2	sp	FUM	dark olive	tuberculated	dark	normal	inconspicous	obtuse	absent	absent
L16-01	1	capillaris	BAR, ALE	pale gray	absent	pale	normal	inconspicous	acute	absent	present
L16-02	2	implexa	PSO	brown	fissural	medium	normal	conspicous	obtuse	absent	absent
L16-03	2	implexa	PSO	pale grayish brown	fissural and tuberculated	pale	paler	conspicous	obtuse	absent	absent
L16-04	2	fuscescens	NOR, PSO	dark brown	fissural	dark	paler	inconspicous	mixed	absent	absent
L16-05	2	fuscescens	NOR, PSO	pale brown	absent	pale	normal	inconspicous	mixed	absent	absent
L16-06	1	capillaris	NOR, PSO	nearly white	absent	pale	normal	inconspicous	acute	absent	absent
L16-07	2	fuscescens	NOR, PSO	very dark grayish brown	absent	dark	normal	conspicous	acute	absent	absent
L16-08	2	implexa	PSO	pale brown	tuberculated	pale	normal	conspicous	mixed	absent	absent
L16-09	2	implexa	PSO	brown	tuberculated	medium	normal	conspicous	mixed	absent	absent
L16-10	2	fuscescens	PSO	very dark olive	absent	dark	normal	conspicous	acute	absent	absent
L16-11	1	capillaris	BAR, ALE	nearly white	absent	pale	normal	inconspicous	mixed	absent	absent
L16-12	2	fuscescens	PSO	dark brown	absent	dark	paler	inconspicous	acute	absent	absent
L16-14	1	capillaris	BAR, ALE, PSO	pale gray	absent	pale	normal	inconspicous	mixed	absent	absent
L16-15	2	sp	PSO	brown	absent	medium	normal	conspicous	acute	absent	absent
L16-16	2	sp	PSO	pale grayish brown	fissural	pale	normal	inconspicous	mixed	absent	absent
L16-17	2	sp	NOR, PSO	pale grayish brown	fissural and tuberculated	pale	normal	conspicous	mixed	present	absent
L16-18	2	sp	PSO	gray	tuberculated	medium	normal	inconspicous	mixed	absent	absent
L16-19	1	capillaris	BAR, ALE, PSO	nearly white	absent	pale	normal	inconspicous	acute	absent	absent
L16-20	1	capillaris	BAR, ALE, PSO	pale yellowish gray	absent	pale	normal	inconspicous	mixed	absent	absent
L16-21	1	capillaris	BAR, NOR, ALE, PSO	pale gray	absent	pale	normal	inconspicous	mixed	absent	absent
L17-01	2		NO SUBS		tuberculate			conspicuous		absent	absent
L17-02	2		ALE, BAR		absent			inconspicuous		absent	absent
L17-03	2		ALE, BAR, FUM		tuberculate			conspicuous		absent	absent
L17-04	2		FUM		tuberculate			inconspicuous		absent	absent
L17-05	2		FUM		tuberculate			inconspicuous		absent	absent
L17-06	2		FUM		tuberculate			conspicuous		absent	absent
L17-07	2		FUM		tuberculate			conspicuous		absent	absent
L17-08	2		FUM		tuberculate			conspicuous		absent	absent
L17-09	2		FUM		tuberculate			inconspicuous		absent	absent
L17-10	2		ALE, BAR		absent			inconspicuous		present	absent
L17-11	2		FUM		tuberculate			conspicuous		absent	absent
L17-12	2		ALE		absent			conspicuous		absent	absent
L17-13	2		FUM		absent			conspicuous		absent	absent
L17-14	2		ALE		absent			inconspicuous		absent	absent
L18-01	2		NOR		absent			conspicuous		absent	absent
L18-02	2		ALE, BAR		absent			conspicuous		absent	absent
L18-03	1		ALE, BAR		absent			inconspicuous		absent	absent
L18-04	2		NOR		absent			inconspicuous		present	absent

L18-05	2	ALE, BAR	absent	inconspicuous	absent	absent
L18-06	1	ALE, BAR	absent	conspicuous	present	absent
L18-07	2	NOR	absent	inconspicuous	absent	absent
L18-08	2	ALE, BAR	absent	inconspicuous	absent	absent
L18-09	2	NO SUBS	absent	conspicuous	absent	absent
L18-10	1	ALE, BAR	absent	inconspicuous	absent	absent
L18-11	1	ALE, BAR	absent	inconspicuous	absent	absent
L18-12	2	ALE, BAR, NOR	absent	inconspicuous	absent	absent
L18-13	1	ALE, BAR	absent	inconspicuous	absent	absent
L18-14	2	GYR, NOR	absent	conspicuous	absent	absent
L18-15	1	ALE, BAR	absent	inconspicuous	absent	absent
L18-16	2	NOR	absent	inconspicuous	absent	absent
L18-17	1	ALE, BAR	absent	inconspicuous	absent	absent
L18-18	2	NOR	absent	inconspicuous	absent	absent
L18-19	1	ALE, BAR	absent	inconspicuous	absent	absent
L18-20	2	NOR	absent	inconspicuous	absent	absent
L18-21	1	ALE, BAR	absent	inconspicuous	present	absent
L18-22	2	NO SUBS	absent	conspicuous	absent	absent
L18-23	1	ALE, BAR	absent	inconspicuous	absent	absent
L19-01	2	NOR	tuberculate	inconspicuous	absent	absent
L19-02	2	NOR	fissural and tuberculate	conspicuous	absent	absent
L19-03	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L19-04	2	FUM	absent	conspicuous	absent	absent
L19-05	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L19-06	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L19-07	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L19-08	2	FUM	tuberculate	conspicuous	absent	absent
L19-09	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L19-10	2	NOR	fissural	conspicuous	absent	absent
L19-11	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L19-12	2	FUM	fissural and tuberculate	inconspicuous	absent	present
L19-13	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L19-14	2	FUM	absent	conspicuous	absent	absent
L19-15	2	NOR	fissural and tuberculate	conspicuous	absent	absent
L19-16	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L19-17	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L19-18	2	NOR	absent	conspicuous	absent	absent
L19-19	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L19-20	2	NOR	fissural and tuberculate	inconspicuous	absent	absent

L19-21	2	NOR	fissural and tuberculate	conspicuous	absent	absent
L19-22	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L19-23	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L20-01	2	FUM	absent	inconspicuous	absent	absent
L20-02	2	FUM	absent	inconspicuous	absent	absent
L20-03	2	FUM	tuberculate	conspicuous	absent	absent
L20-04	2	FUM	tuberculate	inconspicuous	absent	absent
L20-05	2	FUM	absent	inconspicuous	absent	absent
L20-06	2	FUM	absent	inconspicuous	absent	absent
L20-07	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L20-08	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L20-09	2	NO SUBS	absent	conspicuous	absent	absent
L21-01	2	NO SUBS	tuberculate	inconspicuous	absent	absent
L21-02	2	NOR	absent	inconspicuous	absent	absent
L21-03	2	FUM	absent	conspicuous	absent	absent
L21-04	2	FUM	tuberculate	inconspicuous	absent	absent
L21-05	2	FUM	absent	inconspicuous	absent	absent
L21-06	2	NOR	absent	conspicuous	absent	absent
L21-07	2	FUM	tuberculate	conspicuous	absent	absent
L21-08	2	FUM	absent	conspicuous	absent	absent
L21-09	2	FUM	fissural	conspicuous	absent	absent
L21-10	2	FUM	absent	inconspicuous	absent	absent
L22-01	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-02	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-03	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-04	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-05	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L22-06	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-07	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L22-08	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L22-09	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-10	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-11	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L22-12	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-13	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-14	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-15	2	FUM	fissural and tuberculate	conspicuous	absent	absent

L22-16	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L22-17	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L22-18	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L22-19	2	FUM	absent	conspicuous	present	absent
L22-20	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-21	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-22	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L22-23	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L23-01	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L23-02	2	FUM	tuberculate	conspicuous	absent	absent
L23-03	2	FUM	tuberculate	conspicuous	absent	absent
L23-04	2	FUM	absent	conspicuous	absent	absent
L23-05	2	FUM	tuberculate	conspicuous	absent	absent
L23-06	2	FUM	tuberculate	conspicuous	absent	absent
L23-07	2	FUM	tuberculate	conspicuous	absent	absent
L23-08	2	FUM	tuberculate	conspicuous	absent	absent
L23-09	2	FUM	tuberculate	conspicuous	absent	absent
L23-10	2	FUM	tuberculate	conspicuous	absent	absent
L23-11	2	FUM	tuberculate	conspicuous	absent	absent
L23-12	2	FUM	tuberculate	conspicuous	absent	absent
L23-13	2	FUM	tuberculate	conspicuous	absent	absent
L23-14	2	FUM	tuberculate	conspicuous	absent	absent
L23-15	2	FUM	tuberculate	conspicuous	absent	absent
L23-16	2	FUM	tuberculate	conspicuous	absent	absent
L23-17	2	FUM	tuberculate	conspicuous	absent	absent
L23-18	2	FUM	tuberculate	conspicuous	absent	absent
L23-19	2	FUM	tuberculate	conspicuous	absent	absent
L23-20	2	FUM	tuberculate	conspicuous	absent	absent
L23-21	2	FUM	tuberculate	conspicuous	absent	absent
L23-22	2	FUM	tuberculate	conspicuous	absent	absent
L23-23	2	FUM	tuberculate	conspicuous	absent	absent
L24-01	2	FUM	tuberculate	conspicuous	absent	absent
L24-02	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L24-03	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L24-04	2	FUM	tuberculate	inconspicuous	absent	absent
L24-05	2	FUM	tuberculate	conspicuous	absent	absent
L24-06	2	FUM	tuberculate	conspicuous	absent	absent
L24-07	2	FUM	tuberculate	conspicuous	absent	absent
L24-08	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L24-09	2	FUM	fissural and tuberculate	conspicuous	absent	absent

L25-01	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L25-02	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L25-03	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L25-04	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L25-05	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L25-06	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L25-07	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L25-08	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L25-09	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L25-10	2	FUM	tuberculate	inconspicuous	absent	absent
L25-11	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L25-12	2	FUM	tuberculate	conspicuous	absent	absent
L25-13	2	FUM	tuberculate	conspicuous	absent	absent
L25-14	2	FUM	tuberculate	inconspicuous	absent	absent
L25-15	2	FUM	tuberculate	inconspicuous	absent	absent
L25-16	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L25-17	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L25-18	2	FUM	tuberculate	conspicuous	absent	absent
L25-19	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L25-20	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L25-21	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L25-22	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L25-23	2	FUM	tuberculate	inconspicuous	absent	absent
L26-01	1	UNK	absent	inconspicuous	absent	absent
L26-02	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L26-03	1	ALE, BAR	absent	inconspicuous	absent	absent
L26-04	2	FUM	tuberculate	inconspicuous	absent	absent
L26-05	1	UNK	absent	inconspicuous	absent	absent
L26-06	2	NOR	fissural and tuberculate	conspicuous	absent	absent
L26-07	1	ALE, BAR	absent	inconspicuous	absent	absent
L26-08	2	NOR	tuberculate	conspicuous	absent	absent
L26-09	1	ALE, BAR	absent	inconspicuous	absent	absent
L26-10	2	FUM	fissural and tuberculate	inconspicuous	present	absent
L26-11	1	ALE, BAR	absent	inconspicuous	present	absent
L26-12	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L26-13	2	FUM	absent	conspicuous	present	absent
L26-14	1	ALE, BAR	absent	inconspicuous	absent	absent

L26-15	1	ALE, BAR	absent	conspicuous	absent	absent
L26-16	1	ALE, BAR	absent	inconspicuous	absent	absent
L26-17	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L26-18	1	ALE, BAR	absent	inconspicuous	absent	absent
L26-19	1	ALE, BAR	absent	inconspicuous	present	absent
L26-20	1	ALE, BAR	absent	inconspicuous	absent	absent
L26-21	1	ALE, BAR	absent	inconspicuous	present	absent
L26-22	1	ALE, BAR	absent	inconspicuous	absent	absent
L26-23	2	FUM	fissural and tuberculate	inconspicuous	absent	present
L27-01	2	NO SUBS	absent	inconspicuous	absent	absent
L27-02	2	FUM	fissural	inconspicuous	absent	absent
L27-03	2	FUM	fissural	inconspicuous	absent	absent
L27-04	2	FUM	absent	conspicuous	absent	absent
L27-05	2	FUM	fissural	inconspicuous	absent	absent
L27-06	2	FUM	fissural	inconspicuous	absent	absent
L27-07	2	FUM	absent	inconspicuous	absent	absent
L27-08	2	FUM	absent	inconspicuous	absent	absent
L27-09	2	FUM	tuberculate	inconspicuous	absent	absent
L27-10	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L27-11	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L27-12	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L27-13	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L27-14	2	FUM	absent	inconspicuous	absent	absent
L27-15	2	NO SUBS	absent	inconspicuous	absent	absent
L27-16	2	FUM	absent	inconspicuous	absent	absent
L27-17	2	NO SUBS	absent	inconspicuous	absent	absent
L27-18	2	FUM	tuberculate	inconspicuous	absent	absent
L27-19	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L27-20	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L27-21	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L27-22	2	NO SUBS	absent	inconspicuous	absent	absent
L27-23	2	NO SUBS	absent	inconspicuous	absent	absent
L28-01	1	ALE, BAR	absent	inconspicuous	absent	absent
L28-02	1	ALE, BAR	absent	inconspicuous	absent	absent
L28-03	1	ALE, BAR	absent	conspicuous	absent	absent
L28-04	1	ALE, BAR	absent	conspicuous	absent	absent
L28-05	2	NO SUBS	absent	conspicuous	absent	absent
L28-06	2	NO SUBS	absent	inconspicuous	absent	absent
L28-07	2	NO SUBS	absent	conspicuous	absent	absent
L28-08	2	FUM	tuberculate	conspicuous	absent	absent
L28-09	1	ALE, BAR	absent	inconspicuous	absent	absent
L28-10	1	ALE, BAR	absent	inconspicuous	absent	absent

L28-11	1	ALE, BAR	absent	inconspicuous	absent	absent
L28-12	1	ALE, BAR	absent	inconspicuous	absent	absent
L28-13	1	ALE, BAR	absent	inconspicuous	absent	absent
L28-14	1	ALE, BAR	absent	inconspicuous	absent	absent
L28-15	2	NO SUBS	absent	inconspicuous	absent	absent
L28-16	2	NO SUBS	absent	inconspicuous	absent	absent
L28-17	1	ALE, BAR	absent	conspicuous	absent	absent
L28-18	1	ALE, BAR	absent	conspicuous	absent	absent
L28-19	1	ALE, BAR	absent	conspicuous	absent	absent
L28-20	2	NO SUBS	absent	conspicuous	absent	absent
L28-21	1	ALE, BAR	absent	inconspicuous	absent	absent
L28-22	1	ALE, BAR	absent	conspicuous	absent	absent
L28-23	1	ALE, BAR	absent	inconspicuous	absent	absent
L29-01	1	PSO	absent	conspicuous	absent	absent
L29-02	2	PSO	absent	conspicuous	absent	absent
L29-03	2	PSO	absent	conspicuous	absent	absent
L29-04	2	PSO	absent	conspicuous	absent	absent
L29-05	2	PSO	tuberculate	conspicuous	absent	absent
L29-06	2	PSO	absent	conspicuous	absent	absent
L29-07	1	ALE, BAR, PSO	absent	conspicuous	absent	absent
L29-08	2	PSO	absent	conspicuous	absent	absent
L29-09	2	PSO	absent	conspicuous	absent	absent
L29-10	2	FUM	tuberculate	inconspicuous	absent	absent
L29-11	2	FUM	absent	conspicuous	absent	absent
L29-12	2	PSO	absent	conspicuous	absent	absent
L29-13	2	PSO	absent	conspicuous	absent	absent
L29-14	2	PSO	absent	inconspicuous	absent	absent
L29-15	1	ALE, BAR, PSO	absent	inconspicuous	absent	absent
L29-16	2	PSO	absent	conspicuous	absent	absent
L29-17	2	PSO	absent	conspicuous	absent	absent
L29-18	2	PSO	absent	conspicuous	present	absent
L29-19	1	ALE, BAR, PSO	absent	conspicuous	absent	absent
L29-20	2	PSO	absent	conspicuous	absent	absent
L29-21	2	PSO	absent	conspicuous	absent	absent
L29-22	2	PSO	absent	conspicuous	absent	absent
L29-23	2	PSO	absent	inconspicuous	absent	absent
L30-01	2	PSO	absent	conspicuous	absent	absent
L30-02	2	PSO	absent	inconspicuous	absent	absent
L30-03	2	PSO	absent	conspicuous	absent	absent
L30-04	2	PSO	absent	conspicuous	absent	present
L30-05	2	PSO	absent	conspicuous	absent	absent
L30-06	2	PSO	absent	inconspicuous	absent	absent
L30-07	2	PSO	tuberculate	conspicuous	absent	absent
L30-08	2	PSO	absent	conspicuous	absent	absent
L30-09	2	PSO	absent	conspicuous	absent	absent

L30-10	2	PSO	absent	conspicuous	absent	absent
L30-11	2	PSO	absent	conspicuous	absent	absent
L30-12	2	PSO	absent	conspicuous	absent	absent
L30-13	2	PSO	absent	conspicuous	absent	absent
L30-14	2	PSO	absent	conspicuous	absent	absent
L30-15	2	PSO	absent	conspicuous	absent	absent
L30-16	2	PSO	absent	conspicuous	absent	absent
L30-17	2	PSO	absent	conspicuous	absent	absent
L30-18	2	PSO	absent	conspicuous	absent	absent
L30-19	2	PSO	absent	conspicuous	present	absent
L30-20	2	PSO	absent	conspicuous	absent	absent
L30-21	2	PSO	tuberculate	conspicuous	absent	absent
L30-22	2	PSO	absent	conspicuous	present	absent
L31-01	1	PSO	absent	conspicuous	absent	absent
L31-02	1	PSO	absent	conspicuous	absent	absent
L31-03	2	PSO	absent	conspicuous	absent	absent
L31-04	2	PSO	absent	conspicuous	absent	absent
L31-05	2	PSO	absent	conspicuous	absent	absent
L31-06	1	BAR, PSO	absent	conspicuous	absent	absent
L31-07	1	BAR	absent	inconspicuous	absent	absent
L31-08	2	PSO	absent	conspicuous	absent	absent
L31-09	2	PSO	absent	conspicuous	absent	absent
L31-10	2	PSO	absent	conspicuous	absent	absent
L31-11	2	PSO	absent	conspicuous	absent	absent
L31-12	2	PSO	absent	conspicuous	absent	absent
L31-13	1	NOR	absent	conspicuous	absent	absent
L31-14	1	BAR, PSO	absent	inconspicuous	absent	absent
L31-15	1	ALE, BAR, PSO	absent	inconspicuous	present	absent
L31-16	1	ALE, BAR, PSO	absent	inconspicuous	absent	absent
L31-17	2	PSO	absent	conspicuous	absent	absent
L31-18	2	PSO	absent	conspicuous	absent	absent
L31-19	1	ALE, BAR	absent	inconspicuous	absent	absent
L31-20	1	ALE, BAR, PSO	absent	inconspicuous	absent	absent
L31-21	2	NOR	absent	conspicuous	absent	absent
L31-22	2	PSO	absent	conspicuous	absent	absent
L31-23	1	ALE, BAR, PSO	absent	inconspicuous	absent	absent
L32-01	2	PSO	absent	inconspicuous	absent	absent
L32-02	1	NOR, PSO	absent	inconspicuous	absent	absent
L32-03	2	PSO	absent	inconspicuous	absent	absent
L32-04	2	PSO	absent	inconspicuous	absent	absent
L32-05	2	PSO	absent	conspicuous	absent	absent
L32-07	2	PSO	absent	inconspicuous	absent	absent
L32-08	2	PSO	absent	conspicuous	absent	absent
L32-09	2	NOR	absent	inconspicuous	absent	absent

L32-10	2	PSO	absent	conspicuous	absent	absent
L32-11	2	PSO	absent	conspicuous	absent	absent
L32-12	2	PSO	absent	conspicuous	absent	absent
L32-13	2	PSO	tuberculate	inconspicuous	present	present
L32-14	2	PSO	absent	conspicuous	absent	absent
L32-15	2	PSO	absent	conspicuous	absent	absent
L32-16	2	PSO	absent	conspicuous	absent	absent
L32-17	2	PSO	absent	inconspicuous	absent	absent
L32-19	2	PSO	absent	inconspicuous	absent	absent
L32-20	2	PSO	absent	conspicuous	absent	absent
L32-21	2	PSO	absent	inconspicuous	absent	absent
L32-22	2	PSO	absent	inconspicuous	absent	absent
L32-23	2	PSO	absent	inconspicuous	absent	absent
L33-01	2	FUM	tuberculate	inconspicuous	absent	absent
L33-02	2	FUM	tuberculate	conspicuous	absent	absent
L33-04	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L33-05	2	FUM	tuberculate	conspicuous	absent	absent
L33-06	2	FUM	tuberculate	inconspicuous	absent	absent
L33-08	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L33-09	2	PSO	absent	conspicuous	absent	absent
L33-10	2	PSO	absent	conspicuous	absent	absent
L33-11	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L33-12	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L33-13	2	FUM	tuberculate	inconspicuous	absent	absent
L33-14	2	FUM	tuberculate	conspicuous	absent	absent
L33-15	2	FUM	tuberculate	inconspicuous	absent	absent
L33-16	2	FUM	tuberculate	inconspicuous	absent	absent
L33-17	2	FUM	tuberculate	inconspicuous	absent	absent
L33-18	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L33-19	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L33-20	2	FUM	absent	inconspicuous	absent	absent
L33-21	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L33-22	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L33-23	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L34-01	1	ALE, BAR	absent	absent	absent	absent
L34-02	1	ALE, BAR	absent	absent	absent	absent
L34-03	2	FUM	fissural and tuberculate	absent	absent	absent
L34-04	2	FUM	tuberculate	absent	absent	absent
L34-05	2	FUM	absent	absent	absent	absent
L34-06	2	FUM	absent	absent	absent	absent
L34-07	2	FUM	absent	absent	absent	absent

L34-08	2	FUM	tuberculate	absent	absent	absent
L34-09	1	ALE, BAR	absent	absent	absent	absent
L34-11	1	ALE, BAR	absent	absent	absent	present
L34-12	2	FUM	tuberculate	absent	absent	absent
L34-13	2	FUM	absent	absent	absent	absent
L34-14	2	FUM	tuberculate	absent	absent	absent
L34-15	2	FUM	absent	absent	absent	absent
L34-16	1	ALE, BAR	absent	absent	absent	absent
L34-17	1	ALE, BAR	absent	absent	absent	absent
L34-18	1	ALE, BAR	absent	absent	absent	absent
L34-19	1	ALE, BAR	absent	absent	absent	absent
L34-20	2	NO SUBS	absent	absent	absent	absent
L34-21	1	ALE, BAR	absent	absent	absent	absent
L34-22	1	ALE, BAR	absent	absent	absent	absent
L34-23	1	ALE, BAR	absent	absent	absent	absent
L35-01	2	FUM	tuberculate	conspicuous	absent	absent
L35-02	2	FUM	fissural and tuberculate	conspicuous	absent	present
L35-03	2	FUM	absent	conspicuous	absent	present
L35-04	2	FUM	tuberculate	conspicuous	absent	absent
L35-06	2	FUM	absent	inconspicuous	absent	absent
L35-07	2	BAR	absent	conspicuous	present	present
L35-11	2	FUM	tuberculate	conspicuous	present	absent
L35-12	2	FUM	tuberculate	inconspicuous	absent	absent
L35-13	2	BAR	tuberculate	inconspicuous	absent	present
L35-14	2	PSO	absent	conspicuous	absent	absent
L35-15	2	FUM	tuberculate	conspicuous	absent	absent
L35-17	2	NOR	tuberculate	inconspicuous	absent	absent
L35-18	2	BAR	tuberculate	inconspicuous	absent	absent
L35-19	2	FUM	tuberculate	inconspicuous	absent	absent
L35-20	2	FUM	tuberculate	conspicuous	absent	absent
L35-21	2	NO SUBS	absent	inconspicuous	absent	absent
L36-01	2	ALE, BAR, FUM	fissural and tuberculate	inconspicuous	absent	absent
L36-02	2	ALE, BAR, FUM	tuberculate	inconspicuous	absent	absent
L36-03	2	FUM	tuberculate	conspicuous	absent	absent
L36-04	2	FUM	tuberculate	inconspicuous	absent	absent
L36-05	2	ALE, BAR, FUM	fissural and tuberculate	inconspicuous	absent	absent
L36-06	1	ALE, BAR	tuberculate	inconspicuous	absent	absent
L36-07	2	FUM, GYR	tuberculate	conspicuous	absent	absent
L36-08	1	ALE, BAR	absent	inconspicuous	absent	absent
L36-09	2	GYR	tuberculate	conspicuous	absent	absent
L36-10	2	ALE, BAR, FUM	tuberculate	inconspicuous	absent	absent
L36-11	2	FUM	tuberculate	inconspicuous	absent	absent
L36-12	2	ALE, BAR	absent	conspicuous	absent	absent
L36-13	2	ALE, BAR	absent	inconspicuous	absent	absent

L36-14	2	FUM	tuberculate	conspicuous	absent	absent
L36-15	2	PSO	tuberculate	conspicuous	absent	absent
L36-16	2	FUM	tuberculate	inconspicuous	absent	absent
L36-17	2	FUM	tuberculate	inconspicuous	absent	absent
L36-18	2	ALE, BAR, FUM	tuberculate	conspicuous	absent	absent
L36-20	2	GYR	tuberculate	conspicuous	absent	present
L36-21	2	ALE, BAR, FUM	tuberculate	inconspicuous	absent	absent
L36-22	2	FUM, GYR	fissural and tuberculate	inconspicuous	absent	absent
L36-23	2	ALE, BAR, FUM	tuberculate	inconspicuous	absent	absent
L37-01	2	PSO	tuberculate	conspicuous	absent	absent
L37-02	2	FUM	tuberculate	inconspicuous	present	absent
L37-03	2	NOR	absent	inconspicuous	absent	absent
L37-04	2	FUM, PSO	tuberculate	inconspicuous	absent	present
L37-05	2	PSO	absent	conspicuous	absent	absent
L37-06	2	FUM	absent	conspicuous	absent	absent
L37-07	2	PSO	absent	conspicuous	absent	absent
L37-08	2	PSO	absent	conspicuous	absent	absent
L37-09	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L37-10	1	BAR	absent	inconspicuous	absent	absent
L37-11	2	PSO	absent	conspicuous	absent	absent
L37-12	2	PSO	absent	conspicuous	absent	absent
L37-13	2	PSO	absent	conspicuous	absent	absent
L37-14	2	PSO	absent	conspicuous	absent	absent
L37-15	2	PSO	absent	inconspicuous	present	absent
L37-16	2	FUM	absent	conspicuous	present	absent
L37-17	2	FUM	absent	conspicuous	absent	absent
L37-18	2	FUM	tuberculate	inconspicuous	absent	absent
L37-19	2	PSO	tuberculate	conspicuous	absent	absent
L37-20	2	FUM	tuberculate	conspicuous	absent	absent
L37-21	2	FUM	absent	conspicuous	present	present
L37-22	2	FUM	tuberculate	inconspicuous	present	absent
L37-23	2	PSO	absent	conspicuous	absent	absent
L37-24	2	FUM	tuberculate with spinules	inconspicuous	absent	absent
L38-01	2	FUM	absent	inconspicuous	absent	absent
L38-02	2	FUM	tuberculate	inconspicuous	absent	absent
L38-03	2	FUM	tuberculate	inconspicuous	absent	absent
L38-04	2	FUM	tuberculate	inconspicuous	absent	absent
L38-05	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L38-06	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L38-07	2	FUM	tuberculate	conspicuous	absent	absent
L38-08	2	FUM	tuberculate	inconspicuous	absent	absent
L38-09	2	FUM	absent	conspicuous	absent	absent

L38-10	2	FUM	fissural	conspicuous	absent	absent
L38-11	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L38-12	2	FUM	tuberculate	inconspicuous	absent	absent
L38-13	2	FUM	tuberculate	inconspicuous	absent	absent
L38-14	2	FUM	tuberculate	inconspicuous	absent	absent
L38-15	2	No Subs	tuberculate	inconspicuous	absent	absent
L38-16	2	FUM	tuberculate	inconspicuous	absent	absent
L38-17	2	FUM	absent	inconspicuous	absent	absent
L38-18	2	FUM	tuberculate	inconspicuous	absent	absent
L38-19	2	FUM	tuberculate	inconspicuous	absent	absent
L38-20	2	FUM	tuberculate	inconspicuous	absent	absent
L38-21	2	FUM	absent	inconspicuous	absent	absent
L38-22	2	FUM	absent	inconspicuous	absent	absent
L38-23	2	FUM	absent	inconspicuous	absent	absent
L38-24	2	FUM	absent	inconspicuous	absent	absent
L38-25	2	FUM	tuberculate	conspicuous	absent	absent
L39-01	2	FUM	tuberculate	inconspicuous	absent	absent
L39-02	2	FUM	fissural	inconspicuous	absent	absent
L39-03	2	FUM	tuberculate	inconspicuous	absent	absent
L39-04	2	FUM	tuberculate	inconspicuous	absent	absent
L39-05	2	FUM	tuberculate	inconspicuous	absent	absent
L39-06	2	FUM	tuberculate	conspicuous	absent	absent
L39-07	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L39-08	2	FUM	tuberculate	inconspicuous	absent	absent
L39-09	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L39-10	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L39-11	2	FUM	absent	inconspicuous	absent	absent
L39-12	2	FUM	absent	conspicuous	absent	absent
L39-13	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L39-14	2	FUM	tuberculate	inconspicuous	absent	absent
L39-15	2	FUM	tuberculate	inconspicuous	absent	absent
L39-16	2	FUM	absent	inconspicuous	absent	absent
L39-17	2	FUM	absent	inconspicuous	absent	absent
L39-18	2	FUM	absent	inconspicuous	absent	absent
L39-19	2	FUM	tuberculate	inconspicuous	absent	absent
L39-20	2	FUM	tuberculate	inconspicuous	absent	absent
L39-21	2	FUM	tuberculate	inconspicuous	absent	absent
L40-01	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-02	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-03	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-04	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-05	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-06	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-07	1	ALE, BAR	absent	inconspicuous	absent	absent

L40-08	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-09	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-10	2	FUM	fissural	inconspicuous	absent	absent
L40-11	2	NOR	fissural and tuberculate	inconspicuous	absent	absent
L40-12	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-13	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L40-14	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-15	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-17	2	NOR	fissural and tuberculate	inconspicuous	absent	absent
L40-18	1	ALE, BAR	absent	inconspicuous	absent	absent
L40-19	1	ALE, BAR	absent	conspicuous	absent	absent
L40-20	2	NOR	absent	conspicuous	absent	absent
L40-21	1	ALE, BAR	absent	inconspicuous	absent	absent
L41-01	2	PSO	absent	conspicuous	absent	absent
L41-02	2	PSO	fissural	inconspicuous	absent	absent
L41-03	2	PSO	fissural	conspicuous	absent	absent
L41-04	2	PSO	absent	inconspicuous	absent	absent
L41-05	2	PSO	absent	conspicuous	absent	absent
L41-06	2	PSO	fissural	conspicuous	absent	absent
L41-07	2	PSO	tuberculate	conspicuous	absent	absent
L41-08	2	PSO	absent	conspicuous	absent	absent
L41-09	2	PSO	fissural	inconspicuous	absent	absent
L41-10	2	PSO	fissural and tuberculate	inconspicuous	absent	absent
L41-11	2	PSO	tuberculate	conspicuous	absent	absent
L41-13	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L41-14	2	PSO	fissural and tuberculate	conspicuous	absent	absent
L41-15	2	PSO	tuberculate	conspicuous	absent	absent
L41-16	2	PSO	fissural and tuberculate	inconspicuous	absent	absent
L41-17	2	FUM	fissural	inconspicuous	absent	absent
L41-18	2	PSO	tuberculate	conspicuous	absent	absent
L41-19	2	PSO	fissural and tuberculate	conspicuous	absent	absent
L41-20	2	PSO	fissural and tuberculate	conspicuous	absent	absent
L41-21	2	PSO	fissural and tuberculate	conspicuous	absent	absent
L41-22	2	FUM	absent	conspicuous	absent	absent
L42-01	1	ALE, BAR	absent	conspicuous	absent	absent
L42-02	1	ALE, BAR	absent	conspicuous	absent	absent
L42-03	1	ALE, BAR	absent	conspicuous	absent	absent
L42-04	1	ALE, BAR	absent	conspicuous	absent	absent
L42-05	1	ALE, BAR	absent	conspicuous	absent	absent
L42-06	1	ALE, BAR	absent	conspicuous	absent	absent
L42-07	1	ALE, BAR	absent	inconspicuous	absent	absent

L42-08	1	ALE, BAR	absent	inconspicuous	absent	absent
L42-09	1	ALE, BAR	absent	inconspicuous	absent	absent
L42-10	1	ALE, BAR	absent	inconspicuous	absent	absent
L42-11	2	FUM	absent	conspicuous	absent	absent
L42-12	1	ALE, BAR	absent	inconspicuous	absent	absent
L42-13	1	ALE, BAR	absent	inconspicuous	absent	absent
L42-14	2	ALE, BAR	absent	inconspicuous	absent	absent
L42-15	1	ALE, BAR	absent	inconspicuous	absent	absent
L42-16	2	ALE, BAR	absent	inconspicuous	absent	absent
L42-17	2	ALE, BAR	absent	inconspicuous	absent	absent
L42-18	1	ALE, BAR	absent	inconspicuous	absent	absent
L42-19	1	ALE, BAR	absent	inconspicuous	absent	absent
L42-20	2	ALE, BAR	absent	inconspicuous	absent	absent
L42-21	1	ALE, BAR	absent	inconspicuous	absent	absent
L43-01	2	BAR, ALE	absent	inconspicuous	absent	absent
L43-02	2	FUM	tuberculate	conspicuous	absent	absent
L43-03	2	BAR, ALE	tuberculate	inconspicuous	absent	absent
L43-04	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L43-05	2	BAR, ALE	absent	inconspicuous	absent	absent
L43-06	2	BAR, ALE	absent	inconspicuous	absent	absent
L43-07	2	BAR, ALE	absent	inconspicuous	absent	absent
L43-08	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L43-09	2	BAR, ALE	absent	conspicuous	absent	absent
L43-10	2	BAR, ALE	tuberculate	inconspicuous	absent	absent
L43-11	2	FUM	absent	inconspicuous	absent	absent
L43-12	2	BAR, ALE	absent	inconspicuous	absent	absent
L43-13	2	BAR, ALE	absent	inconspicuous	absent	absent
L43-14	2	BAR, ALE	absent	inconspicuous	absent	absent
L43-15	2	BAR, ALE	absent	inconspicuous	absent	absent
L43-16	2	BAR, ALE	absent	inconspicuous	absent	absent
L43-17	2	FUM	absent	conspicuous	absent	absent
L43-18	2	FUM	tuberculate	inconspicuous	absent	absent
L43-19	2	FUM	tuberculate	conspicuous	absent	absent
L43-20	2	BAR, ALE	fissural and tuberculate	inconspicuous	absent	absent
L43-21	2	FUM	absent	conspicuous	absent	absent
L43-22	2	FUM	fissural	conspicuous	absent	absent
L43-23	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L43-24	2	FUM	tuberculate	inconspicuous	absent	absent
L43-25	2	BAR, ALE	tuberculate	inconspicuous	absent	absent
L44-01	2	FUM	fissural	conspicuous	absent	absent
L44-02	2	FUM	fissural	conspicuous	absent	absent
L44-03	2	FUM	fissural	conspicuous	absent	absent
L44-04	2	UNK	fissural	inconspicuous	absent	absent
L44-05	2	FUM	fissural	conspicuous	absent	absent

L44-06	2	UNK	fissural and tuberculate	inconspicuous	absent	absent
L44-07	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L44-08	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L44-09	2	FUM, UNK	tuberculate	inconspicuous	absent	absent
L44-10	2	BAR, ALE	fissural and tuberculate	inconspicuous	absent	absent
L44-11	2	FUM	fissural	conspicuous	absent	absent
L44-12	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L44-13	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L44-14	2	UNK	fissural	inconspicuous	absent	absent
L44-15	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L44-16	2	FUM	tuberculate	inconspicuous	absent	absent
L44-17	2	FUM	tuberculate	inconspicuous	absent	absent
L44-18	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L44-19	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L44-20	2	FUM	tuberculate	inconspicuous	absent	absent
L45-01	2	FUM, ALE, BAR	absent	inconspicuous	absent	absent
L45-02	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-03	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-04	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L45-05	1	ALE, BAR	absent	inconspicuous	absent	absent
L45-06	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-07	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L45-08	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-09	1	ALE, BAR	absent	inconspicuous	absent	absent
L45-10	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-11	2	UNK	tuberculate	inconspicuous	absent	absent
L45-12	2	FUM	absent	inconspicuous	absent	absent
L45-13	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-14	2	ALE, BAR	tuberculate	inconspicuous	absent	absent
L45-15	2	ALE, BAR	tuberculate	inconspicuous	absent	absent
L45-16	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-17	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-18	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-19	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-20	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-21	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-22	2	ALE, BAR	absent	inconspicuous	absent	absent
L45-23	2	UNK	absent	conspicuous	absent	absent
L45-24	2	FUM	absent	conspicuous	absent	absent
L45-25	2	ALE, BAR	absent	inconspicuous	absent	absent

L46-01	2	ALE, BAR	absent	inconspicuous	absent	absent
L46-02	1	ALE, BAR	absent	inconspicuous	absent	absent
L46-03	2	FUM	fissural	conspicuous	absent	absent
L46-04	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L46-05	2	FUM, UNK	fissural	inconspicuous	absent	absent
L46-06	2	ALE, BAR	absent	inconspicuous	absent	absent
L46-07	2	ALE, BAR	absent	inconspicuous	absent	absent
L46-08	2	FUM	absent	conspicuous	absent	absent
L46-09	2	FUM	tuberculate	conspicuous	absent	absent
L46-10	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L46-11	2	FUM	tuberculate	inconspicuous	absent	absent
L46-12	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L46-13	2	ALE, BAR	absent	inconspicuous	absent	absent
L46-14	2	FUM, GYR	tuberculate	conspicuous	absent	absent
L46-15	2	FUM	fissural	conspicuous	absent	absent
L46-17	2	FUM	absent	conspicuous	absent	absent
L46-18	2	GYR	absent	inconspicuous	absent	absent
L46-19	2	FUM	fissural	conspicuous	absent	absent
L46-20	2	ALE, BAR	fissural	inconspicuous	absent	absent
L46-21	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L46-22	2	FUM	fissural	conspicuous	absent	absent
L46-23	1	ALE, BAR	absent	conspicuous	absent	absent
L46-24	2	ALE, BAR	absent	inconspicuous	absent	absent
L47-01	2	ALE, BAR	absent	inconspicuous	absent	absent
L47-02	2	FUM, ALE, BAR	fissural	inconspicuous	absent	absent
L47-03	2	FUM, ALE, BAR	fissural	inconspicuous	absent	absent
L47-04	2	FUM, ALE, BAR	absent	inconspicuous	absent	absent
L47-05	2	FUM, ALE, BAR	fissural	inconspicuous	absent	absent
L47-06	2	FUM	tuberculate	inconspicuous	absent	absent
L47-07	2	ALE, BAR	tuberculate	inconspicuous	absent	absent
L47-08	2	FUM	tuberculate	inconspicuous	absent	absent
L47-09	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L47-10	2	FUM	absent	conspicuous	absent	absent
L47-11	2	GYR	absent	conspicuous	absent	present
L47-12	2	NOR	fissural and tuberculate	inconspicuous	absent	absent
L47-13	2	FUM	tuberculate	conspicuous	absent	absent
L47-14	2	ALE, BAR	absent	inconspicuous	absent	absent
L47-15	1	ALE, BAR	absent	inconspicuous	absent	absent
L47-16	1	ALE, BAR	absent	inconspicuous	absent	absent
L47-17	2	FUM	fissural	conspicuous	absent	absent
L47-18	2	ALE, BAR	tuberculate	inconspicuous	absent	absent

L47-19	2	FUM	tuberculate	inconspicuous	absent	absent
L47-20	2	FUM	tuberculate	inconspicuous	absent	absent
L47-21	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L47-22	2	FUM	tuberculate	conspicuous	absent	absent
L47-23	2	FUM	tuberculate	inconspicuous	absent	absent
L47-24	1	ALE, BAR	absent	inconspicuous	absent	absent
L48-01	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L48-02	2	FUM	tuberculate	inconspicuous	absent	absent
L48-03	2	FUM, ALE, BAR	absent	inconspicuous	absent	absent
L48-04	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L48-05	2	FUM, ALE, BAR, PSO	tuberculate	inconspicuous	absent	present
L48-06	2	FUM	fissural	inconspicuous	absent	absent
L48-07	1	ALE, BAR	absent	conspicuous	absent	absent
L48-08	1	ALE, BAR	absent	inconspicuous	absent	absent
L48-09	2	FUM, ALE, BAR	fissural and tuberculate	inconspicuous	absent	absent
L48-10	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L48-11	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L48-12	1	ALE, BAR	absent	conspicuous	absent	absent
L48-13	2	FUM	tuberculate	inconspicuous	absent	absent
L48-14	2	FUM	fissural	conspicuous	absent	absent
L48-15	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L48-16	2	FUM	fissural	inconspicuous	absent	absent
L48-17	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L48-18	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L48-19	2	FUM, ALE, BAR	fissural	inconspicuous	absent	absent
L48-20	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L48-21	2	FUM	fissural	inconspicuous	absent	absent
L48-22	2	FUM	fissural	inconspicuous	absent	absent
L48-23	2	FUM	fissural	inconspicuous	absent	absent
L49-01	2	FUM	fissural	conspicuous	absent	absent
L49-02	1	ALE, BAR	absent	inconspicuous	absent	absent
L49-03	2	FUM, PSO	tuberculate	inconspicuous	absent	present
L49-04	1	ALE, BAR	absent	inconspicuous	absent	absent
L49-05	1	ALE, BAR	absent	inconspicuous	absent	absent
L49-06	1	ALE, BAR	absent	inconspicuous	absent	absent
L49-07	2	FUM	fissural	conspicuous	absent	absent
L49-08	2	FUM	fissural	conspicuous	absent	absent
L49-09	2	FUM, PSO	absent	inconspicuous	absent	present
L49-10	2	FUM	absent	conspicuous	absent	absent
L49-11	2	FUM	tuberculate	conspicuous	absent	absent

L49-12	2	FUM	tuberculate	conspicuous	absent	absent
L49-13	2	FUM, PSO	fissural and tuberculate	inconspicuous	absent	present
L49-14	2	ALE, BAR	absent	inconspicuous	absent	absent
L49-15	2	FUM	tuberculate	inconspicuous	absent	absent
L49-16	2	ALE, BAR	tuberculate	inconspicuous	absent	absent
L49-17	2	ALE, BAR	fissural	inconspicuous	absent	absent
L49-18	2	ALE, BAR	absent	inconspicuous	absent	absent
L49-19	2	FUM, ALE, BAR	fissural	inconspicuous	absent	absent
L49-20	2	ALE, BAR, PSO	absent	inconspicuous	absent	present
L49-21	2	ALE, BAR	absent	inconspicuous	absent	absent
L49-23	3	ALE, BAR	absent	inconspicuous	absent	absent
L49-24	2	FUM	absent	conspicuous	absent	absent
L49-25	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L50-01	1	ALE, BAR	absent	inconspicuous	absent	absent
L50-02	2	FUM, PSO	tuberculate	conspicuous	absent	present
L50-03	2	FUM, PSO	absent	conspicuous	present	present
L50-04	2	ALE, BAR	fissural	inconspicuous	absent	absent
L50-05	2	FUM	tuberculate	inconspicuous	absent	absent
L50-06	2	FUM, PSO	tuberculate	conspicuous	present	present
L50-07	2	FUM	fissural and tuberculate	conspicuous	present	absent
L50-08	2	FUM	tuberculate	conspicuous	absent	absent
L50-09	2	FUM	tuberculate	conspicuous	present	absent
L50-10	2	FUM	fissural	conspicuous	present	absent
L50-11	2	FUM	fissural	conspicuous	present	absent
L50-12	2	FUM	fissural and tuberculate	inconspicuous	present	absent
L50-13	1	ALE, BAR	absent	inconspicuous	present	absent
L50-14	2	FUM	tuberculate	inconspicuous	present	absent
L50-15	2	FUM	fissural and tuberculate	inconspicuous	present	absent
L50-16	2	FUM, PSO	tuberculate	inconspicuous	present	present
L50-17	2	FUM, PSO	tuberculate	conspicuous	present	present
L50-18	2	FUM, ALE, BAR	absent	inconspicuous	absent	absent
L50-19	2	FUM	fissural	conspicuous	absent	absent
L50-20	2	FUM	tuberculate	conspicuous	absent	absent
L50-21	2	FUM, PSO	tuberculate	conspicuous	present	present
L50-22	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L50-23	2	FUM	fissural	conspicuous	present	absent
L50-24	2	ALE, BAR	absent	inconspicuous	absent	absent
L50-25	2	FUM, PSO	fissural	conspicuous	present	present
L51-01	1	ALE, BAR	absent	conspicuous	absent	absent
L51-02	2	FUM	fissural	conspicuous	present	absent
L51-04	1	ALE, BAR	absent	inconspicuous	present	absent
L51-05	2	ABS	absent	inconspicuous	present	absent

L51-06	2	FUM	fissural	inconspicuous	present	absent
L51-07	2	ALE, BAR	tuberculate	inconspicuous	absent	absent
L51-08	1	ALE, BAR	absent	inconspicuous	absent	absent
L51-09	1	ALE, BAR	absent	inconspicuous	absent	absent
L51-10	1	ALE, BAR	absent	inconspicuous	absent	absent
L51-11	2	FUM	fissural and tuberculate	inconspicuous	present	absent
L51-12	2	FUM, GYR	fissural	conspicuous	absent	absent
L52-01	2	FUM	fissural	conspicuous	absent	absent
L52-02	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-03	2	FUM	fissural	inconspicuous	absent	absent
L52-04	2	FUM	fissural	inconspicuous	absent	absent
L52-05	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L52-06	2	FUM	tuberculate	inconspicuous	absent	absent
L52-07	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-08	2	FUM	tuberculate	inconspicuous	absent	absent
L52-09	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-10	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-11	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-12	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-13	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-14	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-15	2	NOR	fissural and tuberculate	conspicuous	absent	absent
L52-16	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-17	2	NOR	fissural and tuberculate	inconspicuous	absent	absent
L52-18	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-19	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L52-20	2	FUM	tuberculate	inconspicuous	absent	absent
L52-21	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-22	2	FUM, NOR	fissural and tuberculate	inconspicuous	absent	absent
L52-23	2	FUM, GYR	tuberculate	inconspicuous	absent	absent
L52-24	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L52-25	2	FUM	tuberculate	inconspicuous	absent	absent
L53-01	2	NOR	tuberculate	conspicuous	present	absent
L53-02	2	FUM	tuberculate	conspicuous	absent	absent
L53-03	2	FUM	tuberculate	conspicuous	absent	absent
L53-04	2	FUM	fissural	conspicuous	absent	absent
L53-05	2	NOR	fissural and tuberculate	conspicuous	absent	absent

L53-06	2	FUM, GYR	fissural and tuberculate	conspicuous	absent	absent
L53-07	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L53-08	1	ALE, BAR	absent	conspicuous	absent	absent
L53-09	2	FUM	tuberculate	conspicuous	absent	absent
L53-10	2	GYR	tuberculate	conspicuous	absent	absent
L53-11	2	FUM	tuberculate	inconspicuous	absent	absent
L53-12	2	FUM, GYR	tuberculate	inconspicuous	absent	absent
L53-13	2	FUM	fissural	inconspicuous	absent	absent
L53-14	1	ALE, BAR	absent	inconspicuous	absent	absent
L53-15	2	FUM	tuberculate	conspicuous	absent	absent
L53-16	2	FUM	tuberculate	inconspicuous	absent	absent
L53-17	2	FUM	tuberculate	conspicuous	present	absent
L53-18	2	FUM	tuberculate	inconspicuous	absent	absent
L53-19	1	ALE, BAR	absent	inconspicuous	absent	absent
L53-20	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L53-21	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L53-22	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L53-23	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L53-24	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L53-25	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L54-02	3	ALE, BAR	absent	conspicuous	absent	absent
L54-03	3	ALE, BAR	absent	inconspicuous	absent	absent
L54-04	1	ALE, BAR	absent	inconspicuous	absent	absent
L54-05	2	ALE, BAR	absent	inconspicuous	absent	absent
L54-06	3	ALE, BAR	absent	conspicuous	absent	absent
L54-07	1	ALE, BAR	absent	inconspicuous	absent	absent
L54-08	2	ALE, BAR	absent	inconspicuous	absent	absent
L54-09	1	ALE, BAR	absent	conspicuous	present	absent
L54-10	1	ALE, BAR	absent	inconspicuous	absent	absent
L54-12	1	ALE, BAR	absent	conspicuous	absent	absent
L54-13	1	ALE, BAR	absent	inconspicuous	absent	absent
L54-14	3	ALE, BAR	absent	conspicuous	absent	absent
L54-15	2	GYR	absent	inconspicuous	absent	absent
L54-16	3	ALE, BAR	absent	conspicuous	absent	absent
L54-17	1	ALE, BAR	absent	conspicuous	absent	absent
L54-18	3	ALE, BAR	absent	inconspicuous	absent	absent
L54-19	3	ALE, BAR	absent	conspicuous	absent	absent
L54-20	3	ALE, BAR	absent	inconspicuous	absent	absent
L54-21	1	ALE, BAR	absent	conspicuous	absent	absent
L54-22	2	ALE, BAR	absent	inconspicuous	absent	absent
L54-23	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L54-24	2	GYR	absent	conspicuous	absent	absent

L54-25	1	ALE, BAR	absent	inconspicuous	absent	absent
L55-01	2	FUM	absent	conspicuous	absent	absent
L55-02	2	FUM	fissural	inconspicuous	absent	absent
L55-03	2	FUM	tuberculate	inconspicuous	absent	absent
L55-04	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L55-05	2	FUM	tuberculate	conspicuous	absent	absent
L55-06	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L55-07	2	FUM	tuberculate	conspicuous	absent	absent
L55-08	2	FUM	tuberculate	inconspicuous	absent	absent
L55-09	2	FUM	tuberculate	inconspicuous	absent	absent
L55-10	2	FUM	tuberculate	inconspicuous	absent	absent
L55-12	2	FUM	tuberculate	inconspicuous	absent	absent
L55-13	2	FUM	fissural	inconspicuous	absent	absent
L55-14	2	FUM	tuberculate	conspicuous	absent	absent
L55-15	2	FUM	tuberculate	inconspicuous	absent	absent
L55-16	2	GYR	tuberculate	inconspicuous	absent	absent
L55-17	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L55-18	2	FUM	absent	conspicuous	absent	absent
L55-19	2	FUM	tuberculate	inconspicuous	absent	absent
L55-20	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L55-21	2	FUM	fissural and tuberculate	conspicuous	absent	absent
L55-22	2	FUM	absent	inconspicuous	absent	absent
L55-23	2	FUM	tuberculate	inconspicuous	absent	absent
L55-24	2	FUM	tuberculate	inconspicuous	absent	absent
L55-25	2	FUM	tuberculate	inconspicuous	absent	absent
L56-01	1	ALE, BAR	absent	inconspicuous	absent	absent
L56-02	2	GYR	absent	inconspicuous	absent	absent
L56-03	2	GYR	absent	inconspicuous	absent	absent
L56-05	2	ABS	absent	inconspicuous	absent	absent
L56-07	1	ALE, BAR	absent	inconspicuous	absent	absent
L56-08	2	ALE, BAR	tuberculate	conspicuous	absent	absent
L56-09	1	ALE, BAR	absent	conspicuous	absent	absent
L56-10	1	ALE, BAR	absent	conspicuous	absent	absent
L56-11	2	GYR	tuberculate	conspicuous	absent	absent
L56-12	2	GYR	tuberculate	inconspicuous	absent	absent
L56-13	2	FUM	tuberculate	inconspicuous	absent	absent
L56-14	1	ALE, BAR	absent	inconspicuous	absent	absent
L56-15	2	FUM	absent	inconspicuous	absent	absent
L56-16	1	ALE, BAR	absent	conspicuous	absent	absent
L56-17	2	ABS	absent	inconspicuous	absent	absent
L56-18	1	ALE, BAR	absent	conspicuous	absent	absent
L56-19	1	ALE, BAR	absent	conspicuous	absent	absent
L56-20	2	ABS	absent	inconspicuous	absent	absent

L56-21	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L56-22	1	ALE, BAR	absent	inconspicuous	absent	absent
L56-23	2	ABS	absent	conspicuous	absent	absent
L56-25	2	GYR	tuberculate	inconspicuous	absent	absent
L57-01	1	ALE, BAR	absent	conspicuous	absent	absent
L57-02	1	ALE, BAR	absent	inconspicuous	absent	absent
L57-03	1	ALE, BAR	absent	conspicuous	absent	absent
L57-04	3	ALE, BAR	absent	conspicuous	absent	absent
L57-06	1	ALE, BAR	absent	conspicuous	absent	absent
L57-07	2	ABS	tuberculate	conspicuous	absent	absent
L57-08	1	ALE, BAR	absent	conspicuous	absent	absent
L57-09	2	GYR	tuberculate	inconspicuous	absent	absent
L57-10	1	ALE, BAR	absent	inconspicuous	absent	present
L57-11	2	ALE, BAR	tuberculate	inconspicuous	absent	absent
L57-12	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L57-13	3	ALE, BAR	absent	conspicuous	absent	absent
L57-14	1	ALE, BAR	absent	inconspicuous	absent	absent
L57-15	1	ALE, BAR	absent	inconspicuous	absent	absent
L57-16	2	FUM, GYR	tuberculate	conspicuous	absent	absent
L57-17	2	FUM, GYR	tuberculate	inconspicuous	absent	absent
L57-18	2	FUM	tuberculate	inconspicuous	absent	absent
L57-19	2	FUM, GYR	tuberculate	conspicuous	absent	absent
L57-20	1	ALE, BAR	absent	inconspicuous	absent	absent
L57-21	1	ALE, BAR	absent	inconspicuous	absent	absent
L57-22	1	ALE, BAR	absent	conspicuous	absent	absent
L57-23	2	GYR	tuberculate	conspicuous	absent	absent
L57-24	1	ALE, BAR	absent	inconspicuous	present	absent
L57-25	2	FUM	tuberculate	inconspicuous	absent	absent
L58-01	2	FUM	tuberculate	inconspicuous	absent	absent
L58-02	2	ABS	absent	conspicuous	absent	absent
L58-03	2	ABS	absent	inconspicuous	absent	absent
L58-04	2	FUM	tuberculate	inconspicuous	absent	absent
L58-05	1	ALE, BAR	absent	conspicuous	absent	absent
L58-06	2	FUM, GYR	tuberculate	inconspicuous	absent	absent
L58-07	2	FUM	absent	inconspicuous	absent	absent
L58-08	3	ALE, BAR	absent	conspicuous	absent	absent
L58-09	2	ABS	absent	inconspicuous	absent	absent
L58-10	2	ABS	absent	inconspicuous	absent	absent
L58-12	2	FUM	tuberculate	inconspicuous	absent	absent
L58-13	2	FUM	absent	conspicuous	absent	absent
L58-15	2	ABS	absent	inconspicuous	absent	absent
L58-17	3	ALE, BAR	absent	inconspicuous	absent	absent
L58-18	2	GYR	absent	inconspicuous	absent	absent
L58-19	2	GYR	absent	conspicuous	absent	absent
L58-20	2	GYR	absent	conspicuous	absent	absent

L58-21	1	ALE, BAR	absent	conspicuous	absent	absent
L58-22	2	ABS	absent	conspicuous	absent	absent
L58-23	2	ABS	absent	conspicuous	absent	absent
L58-24	2	FUM	tuberculate	inconspicuous	absent	absent
L59-02	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-03	2	FUM	tuberculate	conspicuous	absent	absent
L59-04	2	GYR	absent	inconspicuous	absent	absent
L59-05	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-06	1	ALE, BAR	absent	conspicuous	absent	absent
L59-08	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-10	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-11	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-12	2	GYR	tuberculate	inconspicuous	absent	absent
L59-13	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-14	2	FUM	tuberculate	inconspicuous	absent	absent
L59-15	2	ABS	absent	inconspicuous	absent	absent
L59-16	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-17	2	FUM	tuberculate	inconspicuous	absent	absent
L59-18	1	ALE, BAR	absent	conspicuous	absent	absent
L59-19	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-20	1	ALE, BAR	absent	inconspicuous	present	absent
L59-21	1	ALE, BAR	absent	conspicuous	absent	absent
L59-22	1	ALE, BAR	absent	conspicuous	absent	absent
L59-23	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-24	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-25	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-26	1	ALE, BAR	absent	inconspicuous	absent	absent
L59-27	2	ALE, BAR	tuberculate	inconspicuous	absent	absent
L59-28	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-01	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-02	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-03	2	GYR	tuberculate	inconspicuous	present	absent
L60-04	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-05	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-06	2	GYR	absent	inconspicuous	absent	absent
L60-07	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-08	2	FUM	tuberculate	conspicuous	absent	absent
L60-09	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-10	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-11	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-12	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-13	1	ALE, BAR	absent	inconspicuous	present	absent
L60-14	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-15	2	GYR	absent	conspicuous	absent	absent
L60-17	2	FUM, ALE, BAR	fissural and tuberculate	conspicuous	absent	absent
L60-18	2	ABS	tuberculate	conspicuous	absent	absent

L60-19	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-20	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L60-21	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-22	1	ALE, BAR	absent	conspicuous	absent	absent
L60-23	1	ALE, BAR	absent	inconspicuous	absent	absent
L60-24	2	FUM	absent	inconspicuous	absent	absent
L60-25	2	ABS	tuberculate	inconspicuous	absent	absent
L61-01	3	ALE, BAR	absent	inconspicuous	absent	absent
L61-02	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L61-03	1	ALE, BAR	absent	inconspicuous	present	absent
L61-04	2	ABS	tuberculate	inconspicuous	absent	absent
L61-05	1	ALE, BAR	absent	inconspicuous	absent	absent
L61-06	1	ALE, BAR	absent	inconspicuous	absent	absent
L61-08	2	FUM	tuberculate	inconspicuous	absent	absent
L61-10	3	ALE, BAR	absent	inconspicuous	absent	absent
L61-11	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L61-12	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L61-13	3	ALE, BAR	absent	inconspicuous	absent	absent
L61-14	2	FUM, GYR	tuberculate	inconspicuous	absent	absent
L61-15	2	FUM	fissural	inconspicuous	absent	absent
L61-16	1	ALE, BAR	absent	inconspicuous	absent	absent
L61-17	3	ALE, BAR	absent	conspicuous	absent	absent
L61-18	3	ALE, BAR	absent	inconspicuous	absent	absent
L61-19	1	ALE, BAR	absent	inconspicuous	absent	absent
L61-20	3	ALE, BAR	absent	inconspicuous	present	absent
L61-21	1	ALE, BAR	absent	inconspicuous	present	absent
L61-22	2	FUM	tuberculate	conspicuous	present	absent
L61-24	1	ALE, BAR	absent	inconspicuous	absent	absent
L61-25	1	ALE, BAR	absent	conspicuous	present	absent
L62-01	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-02	2	GYR	absent	inconspicuous	absent	absent
L62-03	1	ALE, BAR	absent	conspicuous	absent	absent
L62-04	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-05	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-06	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-07	2	ALE, BAR	absent	conspicuous	absent	absent
L62-08	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-09	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-10	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-11	1	ALE, BAR	absent	conspicuous	absent	absent
L62-12	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-13	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-14	2	ALE, BAR	absent	inconspicuous	absent	absent
L62-15	1	ALE, BAR	absent	inconspicuous	present	absent

L62-16	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-17	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-18	2	FUM, GYR	fissural	inconspicuous	absent	absent
L62-19	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-20	1	ALE, BAR	absent	conspicuous	absent	absent
L62-21	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-22	1	ALE, BAR	absent	inconspicuous	absent	absent
L62-23	1	ALE, BAR	absent	conspicuous	absent	absent
L62-24	1	ALE, BAR	absent	inconspicuous	absent	absent
L63-01	2	ALE, BAR	absent	conspicuous	absent	absent
L63-02	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L63-03	2	GYR	fissural and tuberculate	conspicuous	absent	absent
L63-04	2	FUM, ALE, BAR	absent	inconspicuous	absent	absent
L63-05	2	ALE, BAR	absent	inconspicuous	absent	absent
L63-06	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L63-07	2	FUM, ALE, BAR	fissural and tuberculate	inconspicuous	absent	absent
L63-08	2	ALE, BAR	tuberculate	inconspicuous	absent	absent
L63-09	2	FUM, GYR	fissural and tuberculate	inconspicuous	absent	absent
L63-10	2	FUM	fissural and tuberculate	inconspicuous	absent	absent
L63-11	2	ALE, BAR	tuberculate	inconspicuous	absent	absent
L63-12	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L63-13	2	ALE, BAR	tuberculate	inconspicuous	absent	absent
L63-14	2	ALE, BAR	fissural	inconspicuous	absent	absent
L63-15	2	ALE, BAR	tuberculate	inconspicuous	absent	absent
L63-16	2	ALE, BAR	absent	inconspicuous	absent	absent
L63-17	2	ALE, BAR	absent	inconspicuous	absent	absent
L63-18	2	FUM	tuberculate	inconspicuous	absent	absent
L63-19	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L63-20	2	FUM, ALE, BAR	tuberculate	inconspicuous	absent	absent
L63-21	2	FUM, ALE, BAR	absent	inconspicuous	absent	absent
L63-22	2	ALE, BAR	absent	inconspicuous	absent	absent
L63-23	2	FUM, ALE, BAR	tuberculate	conspicuous	absent	absent
L63-24	2	ALE, BAR	absent	conspicuous	absent	absent
L63-25	2	ALE, BAR	absent	inconspicuous	absent	absent
L64-01	1	Ale, Bar	absent	conspicuous	absent	absent
L64-02	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-03	1	Ale, Bar	absent	conspicuous	absent	absent
L64-04	1	Ale, Bar	absent	conspicuous	absent	absent
L64-05	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-06	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-07	1	Ale, Bar	absent	conspicuous	absent	absent

L64-08	1	Ale, Bar	absent	conspicuous	absent	absent
L64-09	1	Ale, Bar	absent	conspicuous	absent	absent
L64-10	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-11	1	Ale, Bar	absent	conspicuous	absent	absent
L64-12	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-13	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-14	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-15	1	Ale, Bar	absent	conspicuous	absent	absent
L64-16	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-17	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-18	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-19	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-20	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-21	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-22	1	Ale, Bar, Nor	absent	inconspicuous	absent	absent
L64-23	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-24	1	Ale, Bar	absent	inconspicuous	absent	absent
L64-25	1	Ale, Bar	absent	inconspicuous	absent	absent

Table S8. Results of the Migrate analysis for the selected putative migration routes sowing the posterior distribution of the migration parameter M across all loci, the upper and lower bounds of the 95 % confidence interval and the 2Nm (the product of the M mode and Θ of the recipient population).

Source	Recipient	M Mode	M 2.5 %	M 97.5 %	Recipient Θ	2Nm (M mode × Θ recipient)
Africa	**Canary**	33.67	10.67	57.33	1.28	43.10
Africa	**Iberia**	15.00	0.00	31.33	3.10	46.50
Africa	**Mediterranean**	13.00	0.00	28.67	2.19	28.47
Africa	**Scandinavia**	13.67	0.00	29.33	2.78	38.00
Alps	**Great Britain**	15.00	0.00	30.00	0.51	7.65
Alps	**Carpathians**	25.67	7.33	42.00	2.64	67.77
Alps	**Iberia**	18.33	0.67	35.33	3.22	57.96
Alps	**Mediterranean**	30.33	7.33	82.67	1.03	31.24
Alps	**Scandinavia**	24.33	6.00	42.00	3.73	90.75
Great Britain	**Alps**	9.67	0.00	26.67	2.50	24.18
Great Britain	**Carpathians**	11.00	0.00	27.33	2.87	31.57
Great Britain	**Iberia**	11.00	0.00	27.33	3.18	34.98
Great Britain	**Scandinavia**	11.00	0.00	27.33	3.32	36.52
Canary	**Africa**	15.67	0.00	31.33	0.17	2.66
Carpathians	**Alps**	9.67	0.00	25.33	1.28	12.38
Carpathians	**Great Britain**	19.67	2.00	36.67	0.70	13.77
Carpathians	**Mediterranean**	22.33	2.67	42.00	1.75	39.07
Carpathians	**Scandinavia**	25.00	7.33	42.67	3.21	71.68
Cyprus	**Greece**	18.33	1.33	34.67	2.85	52.24
Greece	**Cyprus**	21.00	0.00	41.33	0.93	19.53
Greece	**Sicily**	13.00	0.00	99.33	0.89	11.57
Iberia	**Africa**	103.00	80.00	124.67	0.53	54.59
Iberia	**Alps**	75.00	55.33	94.67	1.87	140.25
Iberia	**Great Britain**	71.00	46.67	94.67	0.44	31.24
Iberia	**Mediterranean**	55.00	37.33	72.67	0.78	42.9
Iberia	**Scandinavia**	30.33	12.67	47.33	3.06	92.81
Mediterranean	**Africa**	13.67	0.00	29.33	1.86	25.43
Mediterranean	**Alps**	17.00	0.00	33.33	2.14	36.38
Mediterranean	**Carpathians**	13.59	0.00	29.33	2.71	36.83
Mediterranean	**Iberia**	15.00	0.00	31.33	3.17	47.55

Mediterranean	**Scandinavia**	12.33	0.00	28.67	3.92	48.34
Scandinavia	**Africa**	98.33	74.66	121.33	0.25	24.58
Scandinavia	**Alps**	282.33	256.00	308.00	0.46	129.87
Scandinavia	**Great Britain**	27.00	5.33	48.67	0.14	3.78
Scandinavia	**Carpathians**	299.67	275.33	322.67	0.51	152.83
Scandinavia	**Iberia**	145.00	100.67	168.00	0.76	110.2
Scandinavia	**Mediterranean**	837.67	808.00	886.00	0.32	268.05
Sicily	**Greece**	19.67	0.67	37.33	2.23	43.86

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Chapter 6

Molecular studies reveal a new species of *Bryoria* in Chile

Bryoria araucana **and** ***Protousnea*** **spp. growing on an araucaria trunk. Chile, (photo: J. Villagra).**

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Abstract

Bryoria araucana sp. nov. is described from Chile on the basis of morphological, chemical and molecular data. It has a grey to dark greyish brown pendent thallus with the base usually black, branching angles mainly obtuse, terminal branches with few lateral branchlets acutely inserted, fumarprotocetraric acid, and often protocetraric and confumarprotocetraric acids. It is morphologically similar to the Northern Hemisphere *B. trichodes*, but lacks soralia and has inconspicuous concolorous or slightly darker pseudocyphellae. *Bryoria glabra* is also reported for the first time from the Southern Hemisphere. New phylogenetic data based on ITS, mtSSU and MCM7 analyses suggest that *Bryoria* sect. *Bryoria* is polyphyletic and needs revision.

Introduction

Bryoria Brodo & D. Hawksw. is the largest genus in the alectorioid clade of the family Parmeliaceae (Divakar *et al*. 2015), which inhabits temperate to alpine regions worldwide. It has been comprehensively studied in North America and northern Europe (Brodo & Hawksworth 1977; Myllys *et al*. 2011a); however, morphological simplicity and chemical variability make its taxonomy difficult, and recent molecular data have resulted in several changes (Velmala *et al*. 2009, 2014; Myllys *et al*. 2014). Recent studies have discovered additional new species in *Bryoria* from east-central Asia (Myllys *et al.* 2011b; Jørgensen *et al*. 2012), southern South America and the Antarctic (Olech & Bystrek 2004; Fryday & Øvstedal 2012).

Temperate South America is a region with an unexpectedly low reported *Bryoria* diversity, suggesting that additional species may be awaiting discovery in the region. In the course of lichen research by one of us (JV) in the Conguillío National Park in Chile, samples of *Bryoria* growing on *Araucaria araucana* trees were collected. Molecular, morphological and chemical analyses of the specimens revealed the presence of two species**:** *Bryoria glabra* (Motyka) Brodo & D. Hawksw. and another that did not group with any known species in our ongoing morphological, chemical, and phylogenetic analyses. This second species is therefore described here as new.

Table 1. *Bryoria* and *Pseudephebe* specimens used in the study.

Taxon	Specimen	Locality	Chemistry	GenBank accession numbers		
				ITS	mtSSU	MCM7
Bryoria americana	1	Finland, Kainuu	Fum	HQ402677	HQ402636	KJ948017
B. americana	2	Canada, B. C.	Fum, Cfum, Pro	HQ402678	HQ402637	KJ948016
B. araucana	1	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975402**	**KP939085**	**KP975410**
B. araucana*	2	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975405**	**KP939082**	**KP975413**
B. araucana	3	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975404**	**KP939083**	**KP975412**
B. araucana	4	Chile, La Araucaría IX R.	Fum, Cfum,	**KP975403**	**KP939084**	**KP975411**
B. araucana	5	Chile, La Araucaría IX R.	Fum, Cfum	**KP975407**	**KP939081**	**KP975414**
B. araucana	6	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975406**	**KP939080**	**KP975415**
B. bicolor	1	Finland, Etelä-Häme	-	HQ402691	HQ402645	KJ948018
B. bicolor	2	Finland, Koillismaa	Bar, Pso, Fum	HQ402689	HQ402644	KJ948019
B. confusa		China, Yunnan	-	HQ402686	-	KJ948024
B. divergescens		China, Yunnan	Fum, Pro, Cfum, Qua	HQ402705	HQ402654	KJ948025
B. fastigiata		China, Yunnan	Fum, Pro, Cfum	HQ402706	HQ402655	-

B. fremontii	1	Canada, B. C.	No subs	FJ668503	FJ668436	KJ948028
B. fremontii	2	Finland, Koillismaa	Vul in sor.	FJ668498	FJ668432	KJ948029
B. furcellata	1	Finland, Etelä-Savo	Fum, Pro, Cfum	HQ402722	HQ402667	KJ948031
B. furcellata	2	Canada, Manitoba	Fum, Pro, Cfum	HQ402721	HQ402666	KJ948030
B. fuscescens	1	Finland, Koillismaa	Fum, Pro, Cfum	GQ996291	GQ996332	KJ948035
B. fuscescens	2	Finland, Åland	Fum, Pro, Cfum	GQ996290	GQ996322	KJ948032
B. glabra	1	Canada, B. C.	Fum in sor.	HQ402728	HQ402673	KJ948037
B. glabra	2	Finland, Koillismaa	Fum in sor.	FJ668494	FJ668428	KJ948036
B. glabra	7	Chile, La Araucaría IX R.	Fum	**KP975408**	**KP939086**	**KP975417**
B. hengduanensis		China, Yunnan	Usn, Fum, Pro, Cfum	HQ402704	HQ402653	KJ948038
B. lactinea		China, Yunnan	Fum, Pro, Cfum	HQ402699	-	KJ948050
B. nadvornikiana	1	Finland, Kainuu	Bar, Ale, Fum, Cfum, Atr	HQ402718	HQ402663	KJ948053
B. nadvornikiana	2	Iran, East-Azarbaijan	Bar	HQ402720	HQ402665	KJ948052
B. nitidula	1	Sweden, Ångermanland	-	HQ402713	HQ402658	KJ948054
B. nitidula	2	Greenland	Fum, Pro, Cfum	HQ402711	HQ402656	KJ948055
B. poeltii		China, Yunnan	Fum	HQ402701	HQ402650	KJ948057
B. simplicior	1	Finland, Koillismaa	Fatty acids	HQ402714	HQ402659	KJ948063
B. simplicior	2	Russia, Sakha Republic	No subs	HQ402716	HQ402661	KJ948062

B. simplicior	3	Norway, Troms	No subs	**KP975409**	-	**KP975416**
B. smithii	1	Finland, Varsinais-Suomi	No subs	HQ402684	HQ402642	KJ948065
B. smithii	2	India, Uttaranchal	-	HQ402685	HQ402643	KJ948064
B. tenuis	1	Finland, Kainuu	Fum	HQ402694	HQ402648	KJ948074
B. tenuis	2	Sweden, Dalarna	Fum	HQ402695	HQ402649	KJ948073
B. trichodes	1	Canada, Newfoundland	Fum, Cfum, Pro, Atr	HQ402710	-	KJ948075
B. trichodes	2	Russia, Kamchatka	-	KJ947952	-	KJ948076
Pseudephebe pubescens		USA, Alaska	No subs	HQ402676	HQ402635	KJ948091

*Holotype. Newly generated sequences are in bold face. Ale = alectorialic acid, Atr = atranorin, Bar = barbatolic acid, Cfum = confumarprotocetraric acid, Fum = fumarprotocetraric acid, Gyr = gyrophoric acid, Nor = norstictic acid, Pro = protocetraric acid, Pso = psoromic acid, Qua = quaesitic acid, Usn = usnic acid, Vul = vulpinic acid, Unk = unknown, sor = in soralia, and No subs = no lichen substances detected.

Materials and Methods

The specimens collected were analyzed morphologically using standard methods (Smith *et al*. 2009) using a Nikon SMZ-1000 stereomicroscope and a Nikon Eclipse-80i microscope, and photographs were taken with a Nikon 105mm f/2.8D AF Micro-Nikkor lens coupled to a Nikon D90 camera. Spot tests with C, K, KC, and P were carried out as explained in Brodo & Hawksworth (1977). For thin-layer chromatography (TLC), solvents A, B and C were used to run concentrated lichen extracts in 50 °C acetone spotted onto silica gel 60 F254 aluminium sheets (Merck, Darmstadt), according to standard methods (Orange *et al*. 2010). For the best resolution in solvent C, the spotted plate was left to stand for 10 min before running in an acetic acid atmosphere.

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Barcelona) with a slight modification to the manufacturer's instructions (Crespo *et al*. 2001; Divakar *et al*. 2012). Three loci were amplified**:** 1) nrITS, with ITS1FKYO2 (5'-TAG AGG AAG TAA AAG TCG TAA-3') and ITS4KYO2 (5'-RBT TTC TTT TCC TCC GCT-3'; Toju *et al*. 2012) primers; 2) mSSU rDNA, with mtSSU1 (5'-AGC AGT GAG GAA TAT TGG TC-3') and mtSSU3R (5'-ATG TGG CAC GTC TAT AGC CC-3'; Zoller *et al*. 1999) primers; and 3) the low copy protein coding gene MCM7, with MCM71348rev (5'-GAY TTD GCI ACI CCI GGR TCW CCC AT-3') and MCM7-709f (5'-ACI MGI GTI TCV GAY GTH AAR CC-3'; Schmitt *et al*. 2009) primers. For amplification, a reaction mixture of 25 µl was used containing 18 µl sterile water, 2.5 µl ×10 buffer with 2 mM $MgCl_2$, 0.5 µl dNTPs (10 mM of each base), 1.25 µl of each primer at 10 µM, 0.625 µl of DNA polymerase (1 U µl−1), and 1–2 µl DNA template. In failed samples the PCR was repeated using PuReTaq Ready-To-Go PCR Beads (2·5 U of PuReTaq DNA Polymerase, 200 µM of each dNTP, BSA, buffer reaction and stabilizers**:** 10 mM Tris-HCl ph 9.0, 50 mM KCl, 1.5 mM $MgCl_2$; GE Healthcare, Little Chalfont, UK) adding to the lyophilized bead 20µl of sterile water, 1 µl of each primer at 10 µM, and 1·5 µl of DNA template.

Amplifications were run in an automatic thermocycler (XP Cycler, Bioer, Hangzhou) using the following parameters for ITS rDNA and mtSSU rDNA**:** initial denaturation 5 min at 95 °C, then 35 cycles of 1 min at 95 °C, 1 min at 56 °C, 1.5 min at 72 °C, and a final extension of 10 min at 72 °C. For MCM7 we used a touchdown cycling process**:** initial denaturation 10 min at 94 ºC,then followed by 4 cycles of 45 s at 94 ºC, 50 s at 56 ºC, 1 min at 72 ºC; 4 cycles of 45 s at 94 ºC, 50 s at 54 ºC, 1 min at 72 ºC; 36 cycles of 45 s at 94 ºC, 50 s at 52 ºC, 1min at 72 ºC and a final extension of 8min at 72 ºC. PCR products were cleaned using illustraTM ExoProStar (GE Healthcare, Little Chalfont, UK), according to the manufacturer´s instructions. Sequencing was performed by the Unidad de Genómica (Parque Científico de Madrid).

DNA sequences obtained were manually adjusted using SeqMan version 7.0 (DNAstar, Madison) and MEGA5 (Tamura *et al.* 2011). Some GenBank sequences from Myllys *et al*. (2011b; Table 1) were added to the file and aligned using MAFFT version 7 (Katoh & Standley 2013), with the G-INS-I alignment algorithm, a scoring matrix of 1PAM/k = 2, and 0.1 as offset value. Gblocks version 0.91b (Castresana 2000) was used to delete non-conserved GAPs, allowing smaller final blocks, gap positions within the final blocks, and less strict flanking positions. The alignments of each region and the concatenated one were analyzed using maximum likelihood (ML) and Bayesian (B/MCMC) approaches, with *Pseudephebe pubescens* as outgroup to root the tree (Divakar *et al*. 2015). For maximum likelihood (ML) tree reconstruction, the program RAxML v7.2.8 (Stamatakis 2006) implemented on the Cipres Science Gateway (Miller *et al*. 2010) was used. We selected the GTRGAMMA model, which includes a parameter (Γ) for rate heterogeneity among sites and chose not to include a parameter to estimate the proportion of invariable sites (Stamatakis 2006; Stamatakis *et al*. 2008). Support values were assessed using the 'rapid bootstrapping' option with 1000 replicates. For Bayesian reconstruction, MrBayes v3.2.1 (Ronquist & Huelsenbeck 2003) was used, assuming the general time reversible model (Rodriguez *et al*. 1990) and a discrete gamma distribution with six rate categories (GTR+G). The nucleotide substitution model and parameters were selected using the Akaike Information Criterion as implemented in jModelTest (Posada 2008). A run with four million generations, starting with a random tree and employing eight simultaneous chains, was executed. Every 400th tree was saved to a file. We plotted the log-likelihood scores of sample points against generations using TRACER v.1.5 (http://beast.bio.ed.ac.uk/Tracer) and determined that stationarity had been achieved when the log-likelihood values of the sample points reached an equilibrium value (Huelsenbeck & Ronquist 2001), discarding the trees obtained before stationarity was reached. Posterior probabilities (PPs) were obtained from the 50% majority-rule consensus of sampled trees after excluding the initial 25% as burn-in. The phylogenetic trees were drawn using FigTree v.1.4 (http://tree.bio.ed.ac.uk/software/figtree).

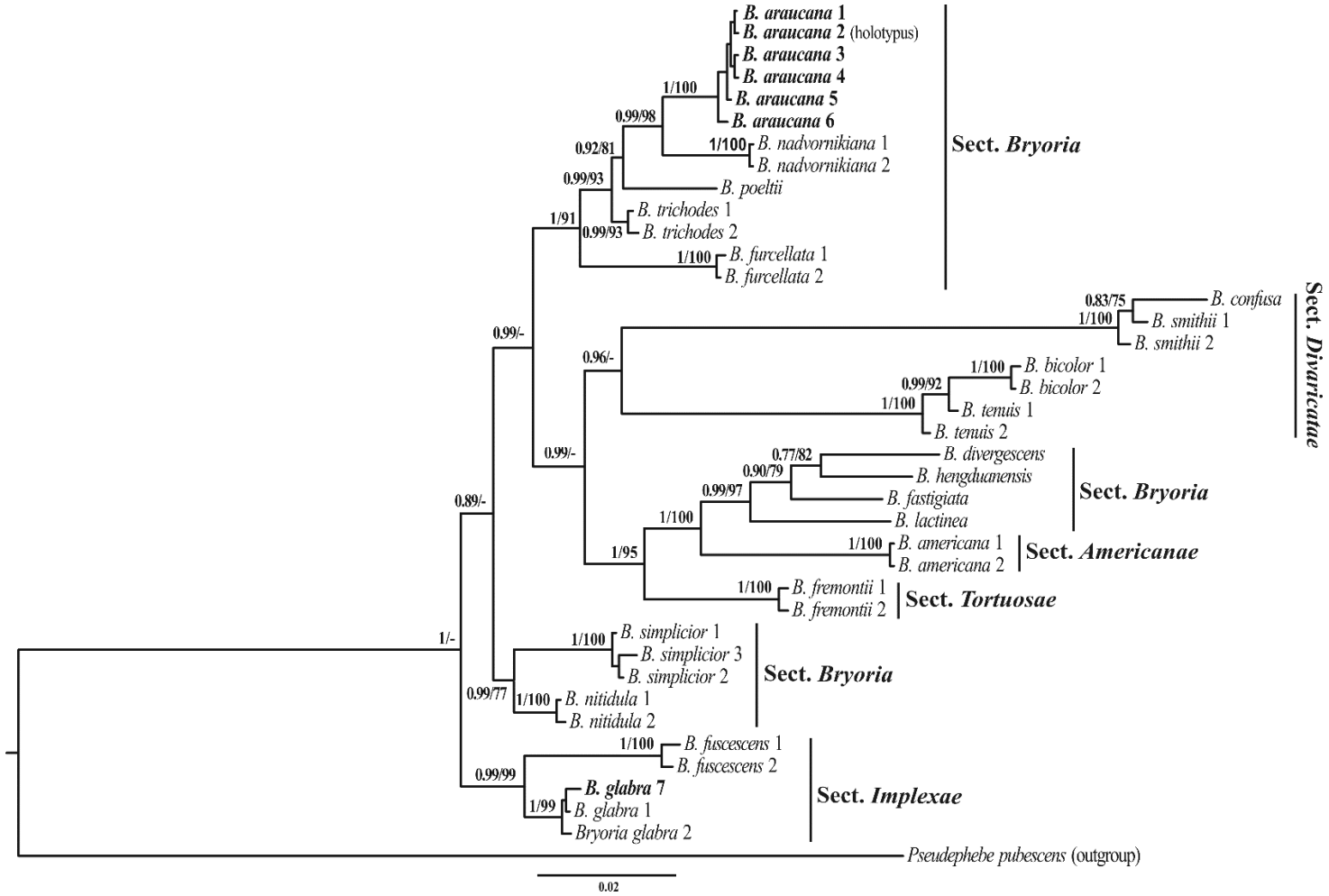

Fig. 1. Phylogenetic relationships of *Bryoria* species used in this study, 38 samples representing 19 species, based on ITS, mtSSU, and MCM7 markers analyzed in a concatenated data matrix. Tree topology depicts the results of the Bayesian Markov chain Monte Carlo (B/MCMC) analysis. Posterior probabilities and bootstrap values, when coincident with the Bayesian tree, are given on the node branches. Sections according to Myllys *et al*. (2011b). *B. glabra* (bold) = Chilean specimen. *B.araucana* (bold) = new species. *Peudephebe pubescens* used as outgroup.

Results and Discussion

The tree obtained from the concatenated ITS, mtSSU and MCM7 dataset (Fig. 1) is mainly based on sequences published by Myllys *et al*. (2011b), who performed a parsimony analysis obtaining five infrageneric sections. Here we subjected those sequences to maximum likelihood and Bayesian analyses, resulting in a different and better supported tree topology. This discrepancy may not be due to the phylogenetic reconstruction method, but to different sampling and the loci used. Sections *Americanae*, *Divaricatae*, *Implexae* and *Tortuosae* are resolved as monophyletic, but in different tree locations than those of Myllys *et al*. (2011b). Section *Implexae* appears as basal rather than derived, and sections *Americanae* and *Tortuosae* are no longer basal. Section *Divaricatae* seems justified, but section *Bryoria* was recovered as polyphyletic and split into three monophyletic groups. In view of this, sections *Americanae*, *Tortuosae* (with one sequenced species each) and *Bryoria* (polyphyletic) will evidently need revision after more detailed analysis has been undertaken. At the species level, *Bryoria tenuis* appears paraphyletic with *B. bicolor*, and *B. smithii* paraphyletic with *B. confusa*, but due to the small number of specimens included in the study it would be premature to propose any change here.

Analyses of the Chilean specimens (Table1; Fig. 1) revealed the presence of *Bryoria glabra*, the first record from the Southern Hemisphere, and a set of different specimens that did not group with any known species. These were phylogenetically close to the Northern Hemisphere *Bryoria nadvornikiana* (Gyeln.) Brodo & D. Hawksw. but they contained fumarprotocetraric rather than barbatolic acid as the main substance. The Chilean specimens were quite different from *B. nadvornikiana* morphologically in that they lacked extensive blackened bases and short, perpendicular, lateral, spinule-like branches. Additionally, they were morphologically and chemically similar to the Northern Hemisphere *Bryoria trichodes* (Michx.) Brodo & D. Hawksw. but lacked soralia, although soralia are not found in every specimen of *B. trichodes*. The material is therefore described here as a new species.

The Species

***Bryoria araucana* Boluda, D. Hawksw. & V. J. Rico sp. nov**.

MycoBank No.**:** MB811960

Resembles the Northern Hemisphere circumboreal *Bryoria trichodes*, but is distinct molecularly, without soralia, and with less conspicuous pseudocyphellae.

Fig. 2. .*Bryoria araucana*, holotype. A, habitat; B, habit; C, detail of branching pattern; D & E, detail of pseudocyphellae. Scales**:** B = 1 cm; C = 1 mm; D = 0.15 mm; E = 0.25 mm.

Type**:** Chile, IX Región de La Araucanía, Provincia de Cautín, Comuna de Melipeuco, Conguillío National Park, Tramo Contrabandistas, Sendero Las Araucarias, close to Conguillío Lake, 38°39'13·57"S, 71° 37'05·27"W, 1215 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 31 August 2014, *J. Villagra* 2 (MAF-Lich. 19718—holotype). GenBank accession numbers**:** KP975405 (ITS), KP939082 (mtSSU), and KP975413 (MCM7).

(Fig. 2)

Thallus pendent to subpendent, 6–12 cm long; isotomic to anisotomic dichotomously branched, angles between dichotomies mainly obtuse, rarely acute; branches terete, even, main branches at base 0.2–0.4 mm of diameter, tips to 0.1 mm of diameter; terminal portions with few lateral branchlets acutely inserted. *Surface* dark grey to dark greyish brown, shiny, base ordinarily black; cortex prosoplectenchymatous. *Soralia* and *isidia* lacking. *Pseudocyphellae* inconspicuous, depressed, fusiform, concolorous to slightly darker than the thallus, sometimes faintly pruinose, straight or twisted, up to 1.5 mm long. *Photobiont* trebouxioid.

Apothecia and *conidiomata* unknown.

Chemistry. Inner cortex and medulla C−, K−, KC −, Pd+ yellow turning red, sometimes faint. TLC**:** fumarprotocetraric acid as the main substance, with protocetraric and confumarprotocetraric acids in trace amounts.

Etymology. Named after the IX Región de la Araucanía in Chile, which is the only known area for the species, as was the case in the name *Araucaria araucana.*

Distribution and ecology. Known only from the type locality and immediate surroundings in the Parque Nacional Conguillío, IX Región de La Araucanía (Chile), occurring on trunks of *Araucaria araucana* in mature open forests (Fig. 2A). Those forests are characteristic of the upper supratemperate bioclimatic belt with the ultraperhumid rainfall regime of the South American Temperate Region (Amigo & Ramírez 1998). Furthermore, the mean annual precipitation in the area, which falls mainly as snow, is c. 2000 mmy^{-1}, and the mean annual temperature is 8.6 °C, with dry and hot short summers (Di Castri & Hajek 1976). *Bryoria araucana* is more frequent on the north-facing trunks exposed to humid winds, growing with *Coelopogon epiphorellus*, *Protousnea dusenii*, *P. magellanica*, *P. poeppigii*, and *Platismatia glauca*. On the south-facing sides of the trunks, it is less frequent, growing with *Nephroma antarcticum*, *Pseudocyphellaria coriifolia*, *P. flavicans*, and *P. granulata*. It may be anticipated that *Bryoria araucana* will be found to have a wider distribution in the temperate forests of the Southern Hemisphere that are almost unexplored for alectorioid lichens.

Conservation status. Although the new species seems not to be frequent, it occurs in a protected area (Parque Nacional Conguillío, Chile). No special actions to conserve the species are currently required.

Additional specimens examined. Bryoria araucana **Chile:** IX Región de La Araucanía, Provincia de Cautín**:** Comuna de Melipeuco, Parque Nacional Conguillío, Tramo Contrabandistas, Sendero Las Araucarias, close to Conguillío Lake, 38°39'14·83"S, 71°37'01·06"W, 1211 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 2013, *J. Villagra* 5 & 6 (MAF-Lich. 19723, 19724); *ibid*., 38°39'13·57"S, 71°37'05·27"W, 1215 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 2014, *J. Villagra* 1, 3 & 4 (MAFLich. 19719, 19720, 19721). *Bryoria glabra* **Chile:** IX Región de La Araucanía, Provincia de Cautín**:** Comuna de Melipeuco, Parque Nacional Conguillío, Tramo Contrabandistas, Sendero Las Araucarias, close to Conguillío Lake, 38°39'13·57"S, 71°37'05·27"W, 1215 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 2014, *J. Villagra* 7 (MAF-Lich. 19722).

Bryoria araucana and the Northern Hemisphere species *B. trichodes* form divergent independent clades which are well supported (Fig. 1). The two species are very similar in morphology and chemistry (*cf*. Brodo & Hawksworth 1977), but *B. araucana* develops less conspicuous pseudocyphellae, lacks atranorin, and apothecia and soralia are unknown. *Bryoria nadvornikiana*, *B. poeltii* (Bystrek) Brodo & D. Hawksw. and *B. furcellata* (Fr.) Brodo & D. Hawksw. are phylogenetically related species, but they can be distinguished by the characters shown in Table 2. Based on the molecular results, restricted distribution, development of inconspicuous pseudocyphellae, and absence of soralia, the new species is well supported.

The *Bryoria glabra* specimen appears to be the first record from the Southern Hemisphere (Fig.1; Table1). It is characterized by repeatedly oval, whitish soralia and regular branching, with rounded and obtuse angles between the branches, and contains fumarprotocetraric acid (Brodo & Hawksworth 1977; Myllys *et al*. 2011b).

Five additional *Bryoria* species are reported in the literature from southern South America (Argentina and Chile)**:** *Bryoria bicolor* (Ehrh.) Brodo & D. Hawksw. (Calvelo & Liberatore 2002), *B. chalybeiformis* (L.) Brodo & D. Hawksw., *B. mariensis* Øvstedaletal. (Fryday & Øvstedal 2012), *B. implexa* (Hoffm.) Brodo & D. Hawksw., and *B. austromontana* P.M. Jørg. & D.J. Galloway (Øvstedal & Lewis Smith 2004). *Bryoria araucana* is distinguished from all these species by the corticolous pendent habit, lack of soralia, branches 0.2– 0.4 mm of diameter, and the sometimes dark basal parts. However, we consider some of these literature records dubious, and in need of verification through molecular analyses.

Table 2. Main diagnostic features for *Bryoria araucana* and phylogenetically related species, based on literature (Brodo & Hawksworth 1977; Bystrek 1969; Myllys *et al*. 2011a; Wang & Chen 1994) and our observations.

Character/Species	*Bryoria araucana*	*B. nadvornikiana*	*B. poeltii*	*B. trichodes*	*B. furcellata*
Main chemistry	Fum	Bar, Ale, ± Atr, Fum in soralia	Fum	Fum, Chlor	Fum
Thallus	Pendent	Caespitose (base) to pendent	Caespitose (base) to pendent	Pendent	Caespitose
Pseudocyphellae	Inconspicuous, dark grey-brown	Inconspicuous, white	Conspicuous, dark brown-black	Conspicuous, white to brownish	Absent
Soralia	Absent	Tuberculate to fissural, white	Tuberculate to fissural, dark, spinulose	Rare, fissural, white	Fissural, white, spinulose (tufts)
Spinules or spinulose branches	On terminal portions, sparse	Lateral, sparse to frequent	Sparse, also on soralia	Lateral, sparse	Sparse to frequent
Colour	Dark grey-brown, base usually darker	Pale to dark brown-violet, base generally black	Dark brown to black	Pale to dark brown	Pale to dark brown, base often darker
Distribution	Chile, South America	Europe, Africa, Asia, Hawaii, North America	Himalayas	Asia, North America	Europe, Macaronesia, Asia, Oceania, North and Central America

Ale = Alectorialic acid, Atr = Atranorin, Bar = Barbatolic acid, Chlor = Chloratranorin, Fum = Fumarprotocetraric acid.

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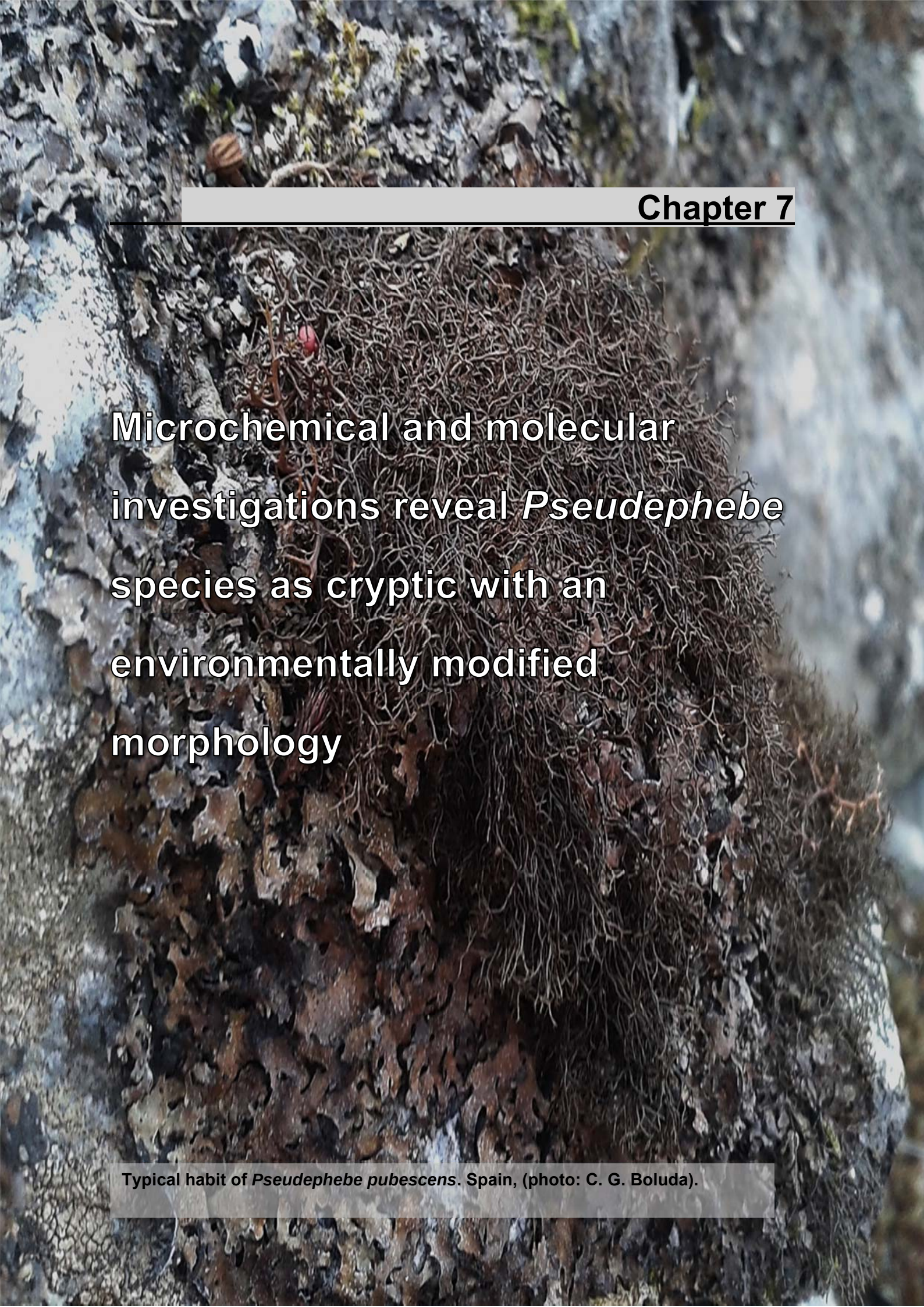

Chapter 7

Microchemical and molecular investigations reveal *Pseudephebe* species as cryptic with an environmentally modified morphology

Typical habit of *Pseudephebe pubescens*. Spain, (photo: C. G. Boluda).

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Abstract

The results of the first molecular phylogenetic study of *Pseudephebe* are presented, a three-locus phylogeny. The genus is confirmed as monophyletic within the Alectorioid clade of *Parmeliaceae*. Two major clades were recovered, which can be assigned to the two traditional taxa, *Pseudephebe minuscula* and *P. pubescens*, with modifications of the species delimitation, especially the variable *P. minuscula*. These species are cryptic and cannot be confidently distinguished morphologically due to phenotypic convergence. Therefore, the use of *P. pubescens* agg. is recommended for non-molecularly analyzed samples. Contrary to previous studies, specimens of both species may have indistinct pseudocyphellae and also contain lichen substances; norstictic acid was detected in *c*. 60 % of specimens tested. An SSU 1516 Group I intron is usually present in *P. minuscula* but always absent in *P. pubescens*. The species-level nomenclature is summarized and sequenced reference specimens (RefSpec) for both *Pseudephebe* species are selected. Sequences from *Bryoria mariensis* established that this name was a synonym of *Pseudephebe minuscula*.

Introduction

Pseudephebe M. Choisy is a genus of lichenized fungi in the alectorioid clade of *Parmeliaceae* (Divakar *et al.* 2015). It is distinguished from the two other genera in the clade with non-septate colourless ascospores, *Bryoria* Brodo & D. Hawksw. and *Nodobryoria* Common & Brodo, primarily by a distinct superficial layer of cells on the cortex (Brodo & Hawksworth 1977). *Pseudephebe* species form small, fruticose to subcrustose cushion-like thalli on hard siliceous rock surfaces and have never been described as producing extrolites (i.e. secondary metabolites). The genus is known from both hemispheres, is circumpolar, and is found in mountains with artic-alpine conditions, from Europe, North America and southern South America (Øvstedal & Smith 2001). It is, however, not known from Africa and is uncommon in Asia and Australia (Wang & McCune 2010; Kantvilas 1994). The genus traditionally comprised two morphologically similar species (Hillmann 1936; Lamb 1964; Brodo & Hawksworth 1977)**:** (1) *Pseudephebe minuscula* (Arnold) Brodo & D. Hawksw. with strongly appressed and tending to be flattened branches, the tips becoming adnate, and with internodes to 1 mm in length; and (2) *P. pubescens* (L.) M. Choisy*,* with terete and never strongly appressed branches, the tips generally free, and internodes 1−3 mm in length. The known distributions of the two species overlap, but *Pseudephebe minuscula* is generally reported from more extreme arctic-alpine habitats than *P. pubescens*, which usually is more common in moister areas (Myllys *et al.* 2011). Both species have a variable morphology, which

has led to the description of many infraspecific taxa, especially forms. These forms have been delimited mainly based on branching and appression degree and the thalli become almost subcrustose in part when growing in the severest environments. Intermediate morphs occur, particularly in extreme habitats, making morphological species delimitation difficult (Imshaug 1957; Brodo & Hawksworth 1977).

Bryoria mariensis, recently described from the Falkland Islands (Fryday & Øvstedal 2012), morphologically resembles large specimens of *Pseudephebe pubescens*. It was described as containing lichenan and having a morphologically similar cortex to *Pseudephebe*; a prosoplechtenchymatous hyphal inner layer and a pseudoparenchymatous surface line with knobby outermost cells. The absence of lichenan excludes a placement in *Nodobryoria* (Common 1991; Common & Brodo 1995). Further, species of *Nodobryoria* also have matt rather than shiny thalli and a knobbly cortex with a jig-saw pattern in surface view. The species was described in *Bryoria*, despite the similarities in cortical structure to *Pseudephebe* species, because of the presence of pseudocyphellae, production of norstictic acid and, what were referred to as soralia; all characters not previously reported in *Pseudephebe* (Fryday & Øvstedal 2012). Given the similarities between *B. mariensis* and *Pseudephebe* we also included the species in our study.

For this study we performed a morphological, microchemical, and a three-locus phylogenetic analyses of a range of *Pseudephebe* specimens and two recently collected *B. mariensis* samples from the type locality.

Materials and Methods

Materials

We studied over 120 *Pseudephebe* specimens from various institutional collections (AAS, BM, K, MAF, MSC, NMW, UBC, and ZT), and the private collection of Nastassja Noell (Reno, Nevada, USA; as hb. N. Noell). Thirty-seven of these were used for phylogenetic reconstruction, with the addition of 25 outgroup specimens (Table 1). Material which was morphologically and microchemically studied, but not molecularly analyzed, is listed according to the names used in the original identification, in Supplementary Material.

Morphology and chemistry

Traditional and additional characters used to distinguish *Pseudephebe pubescens* and *P. minuscula* were examined in all material studied (Table 1, cited under Taxonomy, and in Supplementary Material 1 and 2). For loaned specimens the envelope identification was

maintained, while new collections were identified using Brodo & Hawksworth (1977), Myllys *et al.* (2011) and Smith *et al.* (2009). Specimens were examined morphologically under a Nikon SMZ-1000 stereomicroscope, and hand-cut sections studied with a Nikon Eclipse-80i microscope. Photographs were taken with a Nikon 105 mm f/2.8D AF Micro-Nikkor Lens coupled to a Nikon D90 camera. Spot tests (K, C, and PD) and thin-layer chromatography (TLC) were carried out following Orange *et al.* (2010). We used TLC solvent system C (200 ml toluene / 30 ml acetic acid), with concentrated acetone extracts at 50 ºC spotted onto silica gel 60 F254 aluminium sheets (Merck, Darmstadt). The aluminium sheets were dried for 10 min in an acetic acid atmosphere to maximize resolution. The same lichen fragment used for TLC was used for DNA extraction.

Molecular techniques

DNA was extracted from a single and clean (under a dissecting microscope) lichen branch using the DNeasy Plant Mini Kit (Qiagen, Barcelona) with a slight modification to the manufacturer's instruction (Crespo *et al*. 2001). The fungal nuclear internal transcribed spacer (ITS) rDNA, and partial sequence of the low copy protein coding genes RNA polymerase II largest subunit (*RPB1*) and minichromosome maintenance complex component 7 (MCM7) were amplified using respectively the primers ITS1FKYO2 (5'-TAG AGG AAG TAA AAG TCG TAA-3') and ITS4KYO2 (5'-RBT TTC TTT TCC TCC GCT-3') (Toju *et al.* 2012), RPB1 MH F (5'-ACGTCGCCGAGACCCHAARA-3'; Leavitt *et al*. 2012) and fRPB1-C rev (5'-CCNGCDATNTCRTTRTCCATRTA-3'; Matheny *et al*. 2002) and, Xmcm7 F1 (5'-CGTACACYTGTGATCGATGTG-3'; Leavitt *et al*. 2011) and Mcm7-1348rev (5'-GAYTTDGCIACICCIGGRTCWCCCAT-3'; Schmitt *et al*. 2009). For amplification, we used a reaction mixture of approximately 25 µl, containing 18 µl of sterile water, 2.5 µl of x 10 buffer with 2 mM $MgCl_2$, 0.5 µl dNTPs (10 mM of each base), 1.25 µl of each primer at 10 µM, 0.625 µl of DNA polymerase (1U μl^{-1}), and 0.5-2 µl of DNA elution 2 template. For any failed samples the PCR was repeated using PuRe Taq Ready-To-Go PCR Beads (2.5 U of puReTaq DNA Polymerase, 200 µM of each dNTP, BSA, buffer reaction and stabilizers**:** 10 mM Tris-HCl ph 9.0, 50 mM KCl, 1.5 mM $MgCl_2$; GE Healthcare, Little Chalfont, UK) adding to the lyophilized bead 20 µl of sterile water, 1 µl of each primer at 10 µM and 2 µl of elution 1 DNA template.

Table 1. GenBank accession numbers and species data for specimens used for the phylogenetic tree shown in Fig. 1. Newly obtained sequences are in bold. DNAcode refers to genetic material extracting code, newly obtained extractions preserved in MAF Herbarium DNA-bank. *Pseudephebe* species names are according to the original identifications. Specimens 4591 and 4670 have ITS intrathalline variability, and the alleles with an intron are indicated with (I).

				GenBank Accession numbers		
Species	**Locality**	**Voucher specimen**	**DNAcode**	**ITS**	***RPB1***	***Mcm7***
Alectoria ochroleuca	Austria, Styria	Wedin VIII-1998 (UPS)	MWE 1998	DQ979997	**KU668501**	**KU668455**
A. ochroleuca	Chile, Magallanes	MAF-Lich. 18296	3836	**KU647282**	**KU668502**	**KU668456**
A. sarmentosa	Sweden, Västerbotten	Wedin 6350 (UPS)	6350	DQ979998	DQ923678	KR995525
A. sarmentosa	Spain, Asturias	MAF-Lich. 17914	3710	**KU647283**	**KU668503**	**KU668457**
Allantoparmelia alpicola	Sweden, Lycksele Lappmark	Wedin 7159 (UPS)	MWE 7159	DQ979999	DQ923679	KR995527
Bryocaulon divergens	Sweden, Härjedalen	Odelvik 9145 (S)	MWE 158	**KU647284**	**KU668504**	**KU668458**
B. pseudosatoanum	Japan, Honshu	TNS s. n.	YO 8255	KR995272	KR995448	KR995536
B. satoanum	Japan, Honshu	G. Thor 28135	MWE 163	**KU647285**	**KU668505**	**KU668459**
Bryoria americana	Finland, Kainuu	Velmala 63 (H)	S69	HQ402677	**KU668540**	KJ948017
B. capillaris	Finland, Etelä-Savo	Myllys 485 (H)	L211	GQ996287	**KU668541**	KJ948022
B. capillaris	Finland, Etelä-Häme	Haikonen 22228 (H)	L141	FJ668493	**KU668547**	KJ948020
B. capillaris	Germany, Nordschwarzwald	MAF-Lich. 20111	3879	**KU647286**	**KU668543**	**KU668460**
B. fremontii	Spain, Asturias	MAF-Lich. 18136	3610	**KU647287**	**KU668554**	**KU668461**
B. fremontii	Finland, Etelä-Pohjanmaa	Myllys 490 (H)	L214	FJ668507	**KU668553**	**KU668462**

B. fuscescens	Finland, Oulun-Pohjanmaa	Halonen s. n. (OULU)	L189	GQ996305	**KU668548**	KJ948071
B. fuscescens	Finland, Koillismaa	Velmala 51 & Halonen (H)	S56	GQ996291	**KU668544**	KJ948035
B. glabra	Finland, Koillismaa	Halonen s. n. (OULU)	L186	FJ668494	**KU668549**	KJ948036
B. implexa	Finland, Koillismaa	Velmala *et al.* 23 (H)	S22	GQ996294	**KU668542**	KJ996315
B. implexa	UK, England	Boluda 3855	3855	**KU647288**	**KU668546**	**KU668464**
B. implexa	Iran, East-Azarbaijan	Sohrabi 4656 (H)	L244a	GQ996295	**KU668545**	KJ948042
B. nadvornikiana	Finland, Kainuu	Velmala *et al*. 73 (H)	S79	HQ402718	**KU668550**	KJ948053
B. nadvornikiana	Sweden, Dalarna	Hermansson 14179 (UPS)	L161	HQ402719	**KU668551**	**KU668465**
B. simplicior	Finland, Koillismaa	Velmala *et al*. 30 (H)	S30b	HQ402714	**KU668552**	KJ948063
Gowardia nigricans	Chile, XII Region	MAF-Lich. 18297	3837	**KU647289**	**KU668538**	**KU668466**
G. nigricans	Norway, Troms	Wedin 7297 (UPS)	Wedin 7297	DQ979996	**KU668539**	**KU668467**
Bryoria mariensis	Falkland Islands	NMW.C.2015.004.8	5077	**KU647290**	**KU668519**	**KU668483**
B. mariensis	Falkland Islands	Fryday 10925	5075	**KU647291**	**KU668516**	**KU668480**
Pseudephebe minuscula	USA, Alaska	SRP L-0008791	4339	**KU647292**	**KU668523**	**KU668487**
P. pubescens	USA, Alaska	SRP L-0008806	4338	**KU647293**	**KU668526**	**KU668489**
P. minuscula	USA, Nevada	hb. N. Noell 1442	4784	**KU647294**	**KU668535**	**KU668500**
P. pubescens	USA, California	hb. N. Noell 1581	4781	**KU647295**	**KU668536**	**–**
P. pubescens	USA, Montana	S F175892	4363	**KU647296**	**KU668530**	**KU668493**

P. pubescens	USA, Montana	S F144171	4362	**KU647297**	**KU668528**	**KU668491**
P. pubescens	USA, Oregon	Hollinger 3971	4591	**KU647298**	**KU668529**	**KU668492**
				KX160147 (I)		
P. pubescens	USA, Washington	hb. N. Noell 1557	4783	**KU647299**	**KU668537**	**–**
P. pubescens	Chile, Magallanes	MAF-Lich. 20105	5073	**KU647300**	**KU668517**	**KU668481**
P. pubescens	Chile, Magallanes	MAF-Lich. 20106	5074	**KU647301**	**KU668518**	**KU668482**
P. minuscula	Norway, South Nordland	MAF-Lich. 20107	5079	**KU647302**	**KU668533**	**KU668496**
P. minuscula	Norway, Sogn og Fjordane	MAF-Lich. 20102	5080	**KU647303**	**–**	**KU668497**
P. minuscula	Sweden, Jämtland	S F149958	4360	**KU647304**	**KU668527**	**KU668490**
P. minuscula	Sweden, Jämtland	S F177970	4367	**KU647305**	**KU668524**	**–**
P. pubescens	Sweden, Västerbotten	S F240229	4364	**KU647306**	**KU668520**	**KU668484**
P. pubescens	Austria, Tirol	MAF-Lich. 17091	4201	**KU647307**	**KU668521**	**KU668485**
P. pubescens	Romania, Hunedoara	MAF-Lich. 19475	4668	**KU647308**	**KU668522**	**KU668486**
P. minuscula	Portugal, Beira Baixa	MAF-Lich. 19472	4590	**KU647309**	**KU668525**	**KU668488**
P. minuscula	Portugal, Minho	MAF-Lich. 19473	4670	**KU647310**	**KU668534**	**KU668498**
				KX160146 (I)		
P. pubescens	Spain, Asturias	MAF-Lich. 17838	4199	**KU647311**	**–**	**KU668499**
P.* aff. *minuscula	Spain, Segovia	MAF-Lich. 20103	5071	**KU647312**	**KU668531**	**KU668494**

P.* aff. *minuscula	Spain, Segovia	MAF-Lich. 20104	5072	**KU647313**	**KU668532**	**KU668495**
P. pubescens	Norway, Sogn og Fjordane	MAF-Lich. 20100	5081	**KU647314**	**–**	**KU668475**
P. pubescens	Norway, Sogn og Fjordane	MAF-Lich. 20101	5082	**KU647315**	**KU668512**	**KU668476**
P. pubescens	Norway, South Nordland	MAF-Lich. 20108	5078	**KU647316**	**KU668515**	**KU668479**
P. pubescens	Sweden, Jämtland	S F149572	4365	**KU647317**	**KU668513**	**KU668477**
P. pubescens	Switzerland, Uri	MAF-Lich. 19476	4669	**KU647318**	**KU668511**	**KU668474**
P. pubescens	Spain, Asturias	MAF-Lich. 17907	4198	**KU647319**	**KU668508**	**KU668470**
P. pubescens	Spain, Asturias	MAF-Lich. 17915	4196	**KU647320**	**KU668510**	**KU668472**
P. pubescens	Spain, Asturias	MAF-Lich. 17930	4197	**KU647321**	**KU668514**	**KU668478**
P. pubescens	Spain, Asturias	MAF-Lich. 18112	3709	**KU647322**	**–**	**KU668471**
P. pubescens	Spain, León	MAF-Lich. 19474	4671	**KU647323**	**–**	**KU668473**
P. pubescens	Spain, Teruel	MAF-Lich. 16841	4200	**KU647324**	**KU668509**	**–**
P. pubescens	Spain, Zamora	MAF-Lich. 19470	4589	**KU647325**	**KU668507**	**KU668469**
P. pubescens	Spain, Zamora	MAF-Lich. 19471	3919	**KU647326**	**KU668506**	**KU668468**

Amplifications were run in an automatic thermocycler (XP Cycler, Bioer, Hangzhou, China) using the following parameters: initial denaturation 5 min at 95 ºC, then 35 cycles of 60 s at 95 ºC, 60 s at 56 ºC, 90 s min at 72 ºC, and a final extension of 10 min at 72 ºC for ITS and initial denaturation 10 min at 94 ºC, then followed by 4 cycles of 45 s at 94 ºC, 50 s at 56 ºC, 1 min at 72 ºC; 4 cycles of 45 s at 94 ºC, 50 s at 54 ºC, 1 min at 72 ºC; 36 cycles of 45 s at 94 ºC, 50 s at 52 ºC, 1 min at 72 ºC and a final extension of 8 min at 72 ºC for *RPB1* and *MCM7*. Double bands in an electrophoresis gel of PCR products were isolated using the FavorPrep™ MicroElute Gel / PCR Purification Kit (Favorgen® Biotech, Vienna) following the manufacturer's instructions. PCR products were cleaned using illustra™ ExoProStar (GE Healthcare, Little Chalfont, UK), according to the manufacturer´s instructions. Sequencing was performed by the Unidad de Genómica (Parque Científico de Madrid). DNA sequences obtained were manually adjusted using SeqMan version 7.0 (DNAstar, Madison, USA) and MEGA5 (Tamura *et al.* 2011).

Phylogenetic analyses

Alignments for each locus were performed using MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/; Katoh & Standley 2013) with the G-INS-i alignment algorithm and '1PAM/K = 2' scoring matrix, with an offset value of 0.1, and the remaining parameters set as default. Some *Pseudephebe* specimens contained an intron at 3' end of SSU rDNA, which was removed for the analysis at this level. While alignments for *RPB1* and *MCM7*, were straightforward and did not require manual corrections, exploratory analyses revealed a few ambiguous regions in the ITS alignment. Therefore, we used the program Gblocks v0.91b (Talavera & Castresana 2007) to delimit and remove ambiguous alignment nucleotide positions from the final ITS alignment using the online web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) and implementing the options for a less stringent selection of ambiguous nucleotide positions, including the 'Allow smaller final blocks', 'Allow gap positions within the final blocks', and 'Allow less strict flanking positions' options. The single and concatenated datasets were analyzed using maximum likelihood (ML) and Bayesian (B/MCMC) approaches. To detect topological conflicts among loci, the CADM test (Legendre & Lapointe 2004; Campbell *et al*. 2011) was performed using the function 'CADM.global' implemented in the library "ape" of R (Paradis *et al*. 2004). Analysis resulted in a W of 0.83, which means there is no evidence of well-supported topological conflict. For the maximum likelihood (ML) tree reconstructions, the program RAxML v.7.2.8 (Stamatakis 2006) implemented in Cipres Science Gateway (http://qball2.sdsc.edu:7070/portal2/home.action; Miller *et al.* 2010) was used with the GTRGAMMA model (Stamatakis 2006; Stamatakis *et al*. 2008). Support values were assessed using the "rapid bootstrapping" option with 1000 replicates. For the concatenated dataset, the ITS regions ITS1, 5.8S and ITS2 were partitioned

and for the protein-coding markers we used a three-partition approach, with the first, second, and third codon positions as separate model partitions. For the Bayesian reconstruction, MrBayes v.3.2.1 (Ronquist & Huelsenbeck 2003) was used. Models of DNA sequence evolution for each locus were selected with the program jModeltest2.0 (Darriba *et al*. 2012), using the Akaike information criterion (AIC; Akaike, 1974). The best-fit model of evolution was as follow**:** ITS; ITS1=TIM2+G, 5.8S= K80, ITS2= TIM3ef+G. RPB1; 1st position**:** TIM2+I, 2nd position**:** TrN, 3th position**:** TIM1ef+G. Mcm7; 1st position**:** TPM3, 2nd position**:** F81, 3th position**:** TrNef+G. A run with ten million generations, starting with a random tree and employing twelve simultaneous chains, was executed. Every 500th tree was saved to a file. We plotted the log-likelihood scores of sample points against generations using TRACER v.1.5 (Rambaut *et al*. 2014) and determined that stationarity had been achieved when the log-likelihood values of the sample points reached an equilibrium and ESS values exceeded 200 (Huelsenbeck & Ronquist 2001). Preliminary analysis resulted in a overestimation of branch lengths and to correct this we used the uniform compound Dirichlet prior 'brlenspr=unconstrained:gammadir (1,1,1,1)' (Zamora *et al*. 2015), obtaining rather reasonable branch length estimates. Posterior probabilities (PPs) were obtained from the 50 % majority rule consensus of sampled trees after excluding the initial 25 % as burn-in. The phylogenetic tree was drawn using FigTree v.1.4 (Rambaut 2009).

Species delimitation

In order to establish species limits in the phylogenetic tree, three computational approaches were used**:** (1) Automatic Barcode Gap Discovery ABGD (Puillandre *et al.* 2011) based on barcode gaps using genetic distances; (2) Poison Tree Processes PTP (Zhang *et al.* 2013), based on gene trees; and (3) Bayesian Phylogenetics and Phylogeography BP&P (Yang & Rannala 2010), based on multispecies coalescent model for species validation.

Results

Molecular analysis

GenBank accession numbers of 146 newly obtained sequences are included in Table 1. The final concatenated matrix used as input for the phylogenetic reconstruction contained 1448 unambiguously aligned base pairs (bp) (496 bp for *RPB1*, 458 for *MCM7* and 494 for ITS), with no topological incongruences among loci. Alignments are available in TreeBASE (TB2:S15972). The tree reconstruction (Fig. 1) included all genera belonging to the alectorioid clade (Divakar *et al*. 2015), with the exception of *Nodobryoria* as no DNA-fresh material could

be obtained, and using *Allantoparmelia* (Vain.) Essl. as outgroup. The genera *Alectoria* Ach. (incl. *Gowardia* Halonen *et al.*; see Lumbsch & Huhndorf 2010), *Bryocaulon* Kärnefelt, and *Bryoria* were included to check for any possible alternative affinities for *B. mariensis*. Several species *per* genus were included to facilitate the comparison of species branch lengths with the ones in *Pseudephebe*. *Pseudephebe* was shown to be monophyletic, including *B. mariensis* within it. Within the genus two isolated clades were recovered (A and B; Fig. 1). Clade A is a variable group, containing two major clades, one of them well-supported that we distinguish as Clade A'.

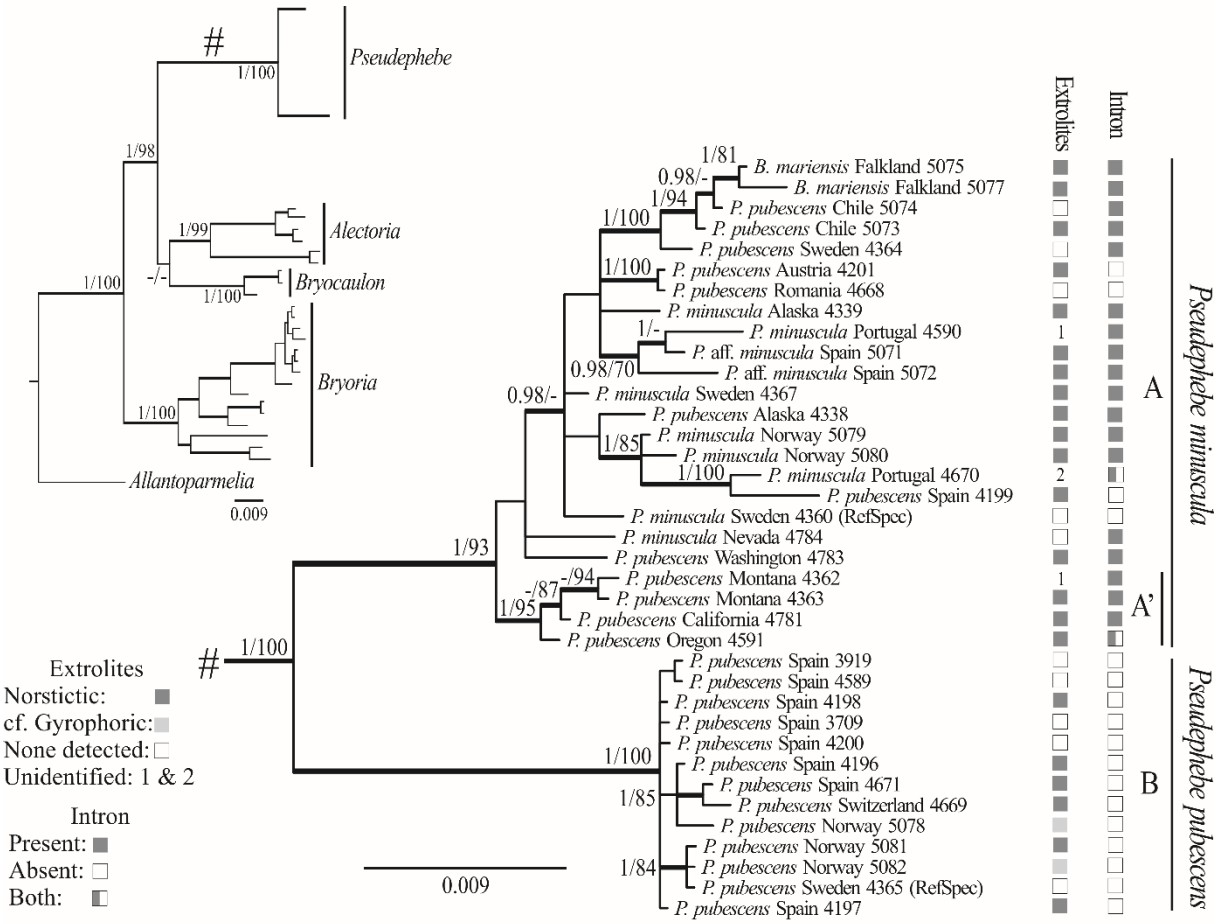

Fig. 1. Phylogenetic consensus tree based on ITS, RPB1 and MCM7 markers analyzed as a concatenated data matrix (Table1). Whole tree in the upper left corner using *Allantoparmelia* as outgroup, with the *Pseudephebe* clade shown in detail.Tree topology depicts the results of Bayesian inference, showing significant posterior probabilities ≥0.95 and bootstrap values ≥70% obtained in the maximum likelihood analysis. Intron presence and extrolite composition are indicated on the lower left corner. RefSpec = sequenced reference specimen.

The region amplified with the primers ITS1FKYO2 and ITS4KYO2 comprises the ITS (ITS1, 5.8 S and ITS2) and a small portion of the 3' end of the SSU gene, which included in some

specimens a group I intron of 233 bp inserted in site 1516 relative to the SSU rDNA sequence of *Escherichia coli* (Gutiérrez *et al*. 2007) or site 1777 of *Saccharomyces cerevisiae*. This intron was detected in many but not all samples in Clade A (Fig. 1), but was absent from all samples in Clade B. In the electrophoresis gel of two samples, two bands corresponding to sequence lengths with and without the intron were distinguished; we checked that result by sequencing each band separately. ITS and SSU are multicopy loci and as DNA have been extracted from a single branch, this result suggests that some copies could contain the intron while others would not.

Morphological and chemical analyses

Morphological and chemically characters of the specimens studied in the phylogenetic analysis are presented in Table 2. The characters traditionally used to distinguish *Pseudephebe minuscula* from *P. pubescens*, i.e., loose or dense appearance, evenly/unevenly thickened branches, branch diameter, internode branch length, and presence of flattened branches, were very variable. The gradient of values found made unequivocal separation into two groups difficult. However, some of the characters studied were taxonomically informative. The presence of a flattened branching pattern correlated with the occurrence of dorsiventrally flattened branches had fewer intermediate states than other characters. The profusion of new ramifications on branch tips (Figs 2−3) was also informative when used together with other characters. Characters related to apothecia, and pycnidia, such as spore size and the presence of marginal cilia, however, could not be adequately assessed as only four of the molecularly analysed *Pseudephebe* specimens had apothecia (see Supplementary Material 2). On those specimens, ciliate apothecia were observed in both clades, and spore measurements were not discriminatory.

Pseudocyphellae, reported here for the first time in the genus, were observed in 23 samples (62%). They varied from inconspicuous to very conspicuous (Fig. 3), and in two samples were perforated. The presence or absence of pseudocyphellae is a taxonomically informative character at the generic level in different clades of *Parmeliaceae* (Elix 1993; Crespo *et al*. 2010). Although reported in the original species description, no true soralia were observed in *Bryoria mariensis*, neither in the type material nor in the additional specimens studied, but frequent short side-branches hapters with globose ends were present.

Pseudephebe is described in all previous literature as lacking detectable extrolites by spot-tests or TLC (Brodo & Hawksworth 1977; Smith *et al*. 2009; Myllys *et al*. 2011). However, about 60% of the studied samples contained norstictic acid, generally in small quantities or in traces, but in some as a major substance. In some specimens, of both *P. pubescens* and *P. minuscula*, we observed the characteristic needle-like red crystals of norstictic acid after the

application of K. The presence of norstictic acid was not correlated with the geographical distribution or any studied morphological character. In addition, a similar spot (with TLC) to gyrophoric acid, and two unidentified substances (substances 1 and 2) were also detected in some samples.

A selection of the most informative characters we found that sometimes can help to distinguish the two *Pseudephebe* species recognized here (i.e. clades A and B) is provided in Table 3.

Table 2. Main characters used to distinguish Pseudephebe species for specimens used for the phylogenetic trees shown in Fig. 1. Pseudephebe species names are according to the original identifications. Internode length and branch width measurements, n = 30, with extreme values in brackets. RefSpec = sequenced reference specimen.

Clade	Species	DNA code	Extrolites	SSU intron	Internodes length (mm)	Branches width (mm)	Pseudo-cyphellae	Profusely branched tips	Old branches appressed	Flattened branches
A	*Bryoria mariensis*	5077	Norstictic	+	< 1−7	0·26 (0·1−1)	+	−	−	−
A	*B. mariensis*	5075	Norstictic	+	< 1−7	0·20 (0·1−0·4)	+	−	±	±
A	*Pseudephebe minuscula*	4339	Norstictic	+	< 1	0·18 (0·1−0·3)	−	+	+	+
A	*P. pubescens*	4338	Norstictic	+	< 1	0·18 (0·1−0·2)	+	+	−	±
A	*P. minuscula*	4784	Absent	+	< 1	0·32 (0·2−0·5; crusty)	+	+	+	+
A'	*P. pubescens*	4781	Norstictic	+	> 1	0·10 (0·1−0·2)	perforated	+	−	−
A'	*P. pubescens*	4363	Norstictic	+	< 1−2	0·17 (0·1−0·3)	±	+	±	+
A'	*P. pubescens*	4362	Greyish pale spot	+	< 1	0·17 (0·1−0·2)	−	+	±	±
A'	*P. pubescens*	4591	Norstictic	±	> 1	0·10 (0·05−0·2)	−	+	−	±

A	*P. pubescens*	4783	Norstictic	+	> 1	0·13 (0·1–0·2)	±	–	–	–
A	*P. pubescens*	5073	Norstictic	+	1–5	0·18 (0·1–0·3)	±	–	–	–
A	*P. pubescens*	5074	Absent	+	> 1	0·16 (0·1–0·3)	±	–	±	–
A	*P. minuscula*	5079	Norstictic	+	< 1–2	0·15 (0·1–0·2)	–	–	+	+
A	*P. minuscula*	5080	Norstictic	+	< 1	0·15 (0·1–0·2)	–	+	+	+
A	*P. minuscula* (RefSpec)	4360	Absent	–	< 1	0·18 (0·1–0·3)	–	+	±	+
A	*P. minuscula*	4367	Norstictic	+	< 1	0·16 (0·1–0·2)	–	+	+	+
A	*P. pubescens*	4364	Absent	+	< 1–1·5	0·17 (0·1–0·3)	±	+	+	+
A	*P. pubescens*	4201	Norstictic	–	< 1–2	0·22 (0·2–0·3)	–	±	±	–
A	*P. pubescens*	4668	Absent	–	< 1	0·15 (0·1–0·2)	–	+	+	+
A	*P. minuscula*	4590	Greyish pale spot	+	< 1–1·5	0·15 (0·1–0·2)	+	+	–	±
A	*P. minuscula*	4670	Brownish spot	±	< 1	0·16 (0·1–0·3)	±	+	±	+
A	*P. pubescens*	4199	Norstictic	–	< 1–2	0·16 (0·1–0·3)	–	±	±	–
A	*P.* aff. *minuscula*	5071	Norstictic	+	> 1	0·20 (0·1–0·3)	+	+	–	–
A	*P.* aff. *minuscula*	5072	Norstictic	+	> 1	0·17 (0·1–0·3)	±	–	–	–
B	*P. pubescens*	5081	Norstictic	–	< 1–1·5	0·15 (0·1–0·2)	–	+	–	±
B	*P. pubescens*	5082	*cf.* Gyrophoric	–	< 1–1·5	0·17 (0·1–0·3)	perforated	–	–	–

B	*P. pubescens*	5078	*cf.* Gyrophoric	−	< 1−2	0·22 (0·1−0·4)	−	−	−	−
B	*P. pubescens* (RefSpec)	4365	Absent	−	> 1	0·16 (0·1−0·2)	−	−	−	−
B	*P. pubescens*	4669	Norstictic	−	> 1	0·17 (0·1−0·3)	−	±	−	−
B	*P. pubescens*	4198	Norstictic	−	> 1	0·17 (0·1−0·3)	+	−	−	−
B	*P. pubescens*	4196	Norstictic	−	> 1	0·17 (0·1−0·3)	+	±	±	−
B	*P. pubescens*	4197	Norstictic	−	> 1	0·19 (0·1−0·3)	+	±	+	±
B	*P. pubescens*	3709	Absent	−	< 1−2	0·14 (0·1−0·2)	±	±	−	−
B	*P. pubescens*	4671	Norstictic	−	> 1	0·17 (0·1−0·2)	±	±	−	−
B	*P. pubescens*	4200	Absent	−	> 1	0·15 (0·1−0·2)	±	−	−	−
B	*P. pubescens*	4589	Absent	−	> 1	0·21 (0·1−0·3)	+	±	−	−
B	*P. pubescens*	3919	Absent	−	> 1	0·21 (0·1−0·3)	+	−	−	−

Species delimitation

Pseudephebe specimens fall into two genetically well-isolated clades (Clades A and B) separated by an unexpectedly long branch (Fig. 1). The traditionally used morphological characters employed to separate these species were not, however, fully congruent with the two clades.

Clade B included specimens of *P. pubescens* conforming to the traditional concept of the species (Brodo & Hawksworth 1977), but also a few which were similar morphologically to *P. minuscula*. On the contrary, 42 % of the specimens from Clade A were originally identified as *P. minuscula*, and the remaining 58 % had been named *P. pubescens* or *Bryoria mariensis*.

Clade A (Fig. 1) contained a much greater genetic variability than Clade B (here assigned to *P. pubescens*), with branch lengths and a topology similar to other alectorioid genera with multiple species (Velmala *et al.* 2014). Subclade A' (Fig. 1), is well supported and comprised specimens from western North America. That material has thin elongated terminal branches that are minutely branched at the end; we initially speculated that this might merit recognition as a separate taxon, but discovered that this character was also evident in other clades.

Species delimitation approaches (Table 4) confirmed the existence of two species as the most probable scenario for the genus as no genetic gap was detected within Clade A (Leavitt *et al.* 2015). We therefore assign Clade A to *Pseudephebe minuscula* and Clade B to *P. pubescens*. Although the two species are morphologically overlapping, an assay with a node datation analysis established that they could have split from a common ancestor around 9.5 to 11.6 Mya (see Supplementary Material 3).

Taxonomy

Where morphological identification cannot be made with confidence, species level identification in *Pseudephebe* should be carried out by molecular methods. In cases where this is not possible, we suggest that collections should be referred to as either *Pseudephebe pubescens* s. l. or *P. pubescens* agg. as discussed by Crespo & Lumbsch (2010). We prefer "agg.", as that term is used in plants particularly in cases covering two or more named species.

In order to leave no doubt in the application of names to the two major clades distinguished here, and for assigning sequences obtained from fresh collections, we designated a sequenced reference specimen for both *Pseudephebe* species.

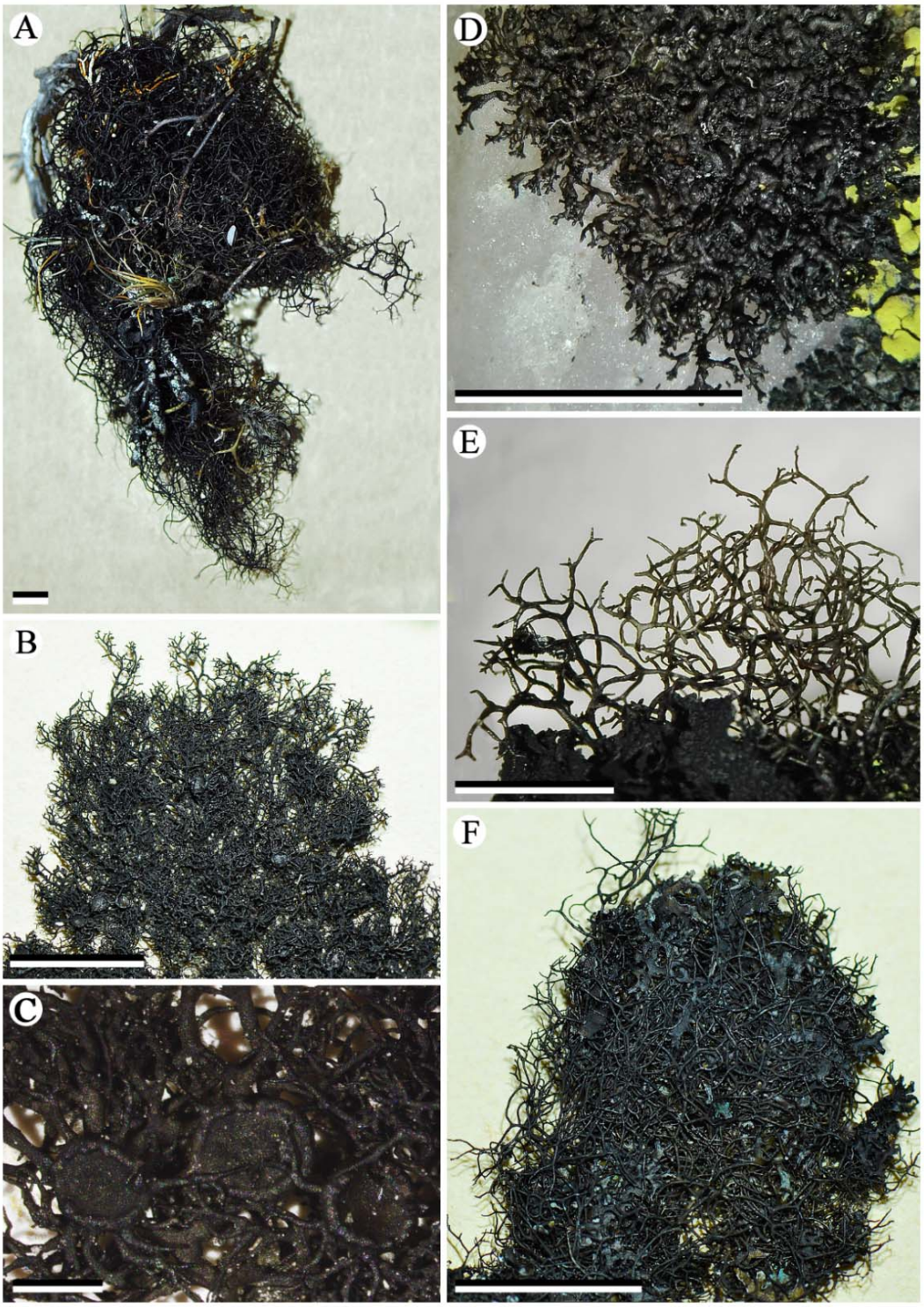

Fig. 2. Variability in *Pseudephebe* species. A–D, *P. minuscula*; A, long branched fruticose habit (NMW. C.2015.004.8); B, short to intermediate, more typical, branched habit (S F149958, sequenced reference specimen); C, specimen with apothecia in detail (S F149958, sequenced reference specimen); D, extreme subcrustose habit (hb. N. Noell 1561). E & F, *P. pubescens*; E, long branched fruticose habit (MAF-Lich. 20100); F, typical habit (S F149572, sequenced reference specimen). Scales**:** A, B, D−F = 5mm; C = 1mm.

We have not re-investigated species names other than those listed below, *viz*. *Alectoria biformis* (Vain.) Dodge, *A. congesta* (Zahlbr.) Dodge, *A. intricata* Hue, and *A*. *nigerrima* Hue, nor the application of numerous infraspecific names employed in the group.

Information on these is provided in Hillman (1936), Lamb (1964, 1948), Hawksworth (1972), Brodo & Hawksworth (1977), and Myllys *et al*. (2011). Many of these names are based on material from Antarctica, and are likely to represent *P. minuscula* as circumscribed here because of the morphology, but ideally should be re-collected and sequenced from the type localities to verify their status.

***Pseudephebe minuscula* (Nyl. *ex* Arnold) Brodo & D. Hawksw.**

Opera Bot. **42:** 140 (1977).—*Imbricaria lanata* var. *minuscula* Nyl. ex Arnold, *Verh. Zool.-Bot. Ges. Wien* **28:** 293 (1878).—*Parmelia minuscula* (Nyl. ex Arnold) Nyl., *Bull. Soc. Linn. Normandie, sér.* 4, **1:** 205 (1887); type**: Finland:** *Lapponia enontekiensis***:** Enontekio, in alpe Pietsovaara prope Kilpisjärvi, 1867, *J. P. Norrlin* (H-NYL 34355—lectotype ?, designated by Brodo & Hawksworth 1977**:** 141 [as ”34255”] but see below); **Sweden:** *Jämtland***:** Undersåker sn, Välliste, topplatån, 1300 m VSV om Norra stugan, 63º16’07”N, 13º08’16.1”E, fjällhed, klippor, på stenblock, 22 July 2009, *G. Odelvik 9152* (S F149958—***reference specimen selected here***, DNAcode 4360; GenBank accession numbers**:** ITS KU647304, RPB1 KU668527, MCM7 KU668490).

Alectoria pacifica Stizenb., *Proc. Calif. Acad. Sci., ser. 2,* **5** (2)**:** 537 (1895)**:** type**:** [**Mexico:**] Insulae californica Guadalupe, supra terram humosam, 1875, *E. Palmer* (ZT Myc55359—holotype; US—isotype n. v.).

Bryoria mariensis Øvstedal *et al.*, *Lichenologist* **44:** 487 (2012); type**: Falkland Islands:** West Falkland, Port Howard, on summit of Mt. María, UTM 21F UC 2079, 2158 ft [658 m], feldmark, 28 January 1968, *H. A. Imshaug 41327 & R. C. Harris* (MSC 80870—holotype).

(Figs. 2A−D & 3A−C, G−M)

Myllys *et al*. (2011**:** 140) designated a different specimen from that selected by Brodo & Hawksworth (1977**:** 141) as lectotype for Nylander's "*minuscula*". They cited an undated specimen from Austria collected by Arnold (H-NYL 34356), without discussion, but presumably as they considered that a lectotype had to be chosen only from material studied by the validating author, in preference to one from Finland collected in 1867 and studied by Nylander (H-NYL 34355) selected by Brodo & Hawksworth. This case is, however, complex as Arnold (1878**:** 293) also referred to earlier usages of the name by Nylander, Stizenberger, and himself. There is no indication Arnold was intending to do anything but cite an identification in a floristic list of species in a region of Austria. Under Art. 9.3, original material in validating descriptions can include materials not seen by the validating author. Also mentioned by Arnold is "Nyl.

Lapp. Or. 120", which is a direct reference to Nylander material, and it seems preferable to have a Finnish specimen as lectotype as Arnold cited Nylander as author. “Lapp. Or. 120” would seem to be a cryptic reference to an exsiccate of Fellman issued in 1865 which, however, has "*Parmelia lanata* f. *minuscula*" as no. 83, while no. 120 is "*Lecanora dicksonii*" (Lynge 1915−16). However, there is a Fellman specimen in H-NYL from “Karelia pomorica orientalis, Suma” dated 1863 (H-NYL 34357) and examined by D.L.H in 1968 which therefore could be a better candidate. The issue merits further study, and a definite resolution of this typification is beyond the scope of the present work. Rather than complicate matters further here, we have indicated a Reference Specimen (RefSpec) to serve as a proxy sequenced type, following the proposal of Ariyawansa *et al*. (2014), as an interim solution to fixing the molecular application of the epithet *minuscula* in our sense.

The name *Alectoria antarctica* Dodge & Baker is excluded here as it was based on a specimen of *Pseudephebe minuscula* infected by a lichenicolous fungus. The lichenicolous fungal element was selected as lectotype for the name by Hawksworth & Iturriaga (2006**:** 202), who transferred it into *Carbonea*.

Specimens referred to *Pseudephebe pubescens* f. *subciliata* (Nyl.) D. Hawksw. forming appressed rosettes and with short internodes appear to belong here. They were retained under *P. pubescens* by Hawksworth (1972) as the lobes were terete rather than dorsiventrally compressed, but from the data presented here that character appears not to be taxonomically informative and environmentally induced. Of the total samples analysed molecularly, specimens from Austria, Chile, the Falkland Islands (Islas Malvinas), Norway, Portugal, Romania, Spain, Sweden, and USA were clustered in the *Pseudephebe minuscula* clade.

Specimens examined (DNA, morphology, and chemistry examined). **Austria:** *Tirol***:** Otzaler Alpen, SE of Martin Busch Haus, 46º47’57”N, 10º52’47”E, 2580 m, on gneissic boulder, 11 August 2011, *R. Türk 49630* [Obermayer*, Lichenoth. Graec.* No. 379] (MAF-Lich. 17091, as *Pseudephebe pubescens*, DNAcode 4201).—**Chile:** *Magallanes y Antártida Chilena (XII Región)***:** Navarino Island, Cabo de Hornos Commune, Bandera Hill, 15 Mar. 2012, *C. Laguna Defior* (MAF-Lich. 20105; DNAcode 5073, TLC**:** norstictic acid; and MAF-Lich. 20106 (DNAcode 5074, TLC**:** no substances detected), 20110).—**Falkland Islands (Islas Malvinas):** *West Falkland (Gran Malvina)***:** Port Howard (Puerto Mitre), Mt. María, 51.60768ºS, 59.58654ºW, 580 m, on sandy soil in feldmark, 26 January 2015, *A. Orange 22484* (NMW s. n., as *Bryoria mariensis*, DNAcode 5077); Mt María summit, *D. Crabtree* [A. Fryday 10925] (MSC, as *Bryoria* sp., DNAcode 5075).—**Norway:** *Sogn og Fjordane***:** Sogn, *c*. Luster, 61º31'58"N, 07º50'49"E, 1316 m, 12 August 2015, on rocky soil with moss and lichens, *C. G. Boluda & N. Calpena* (MAF-Lich. 20101, DNAcode 5082, TLC**:** *cf.* gyrophoric acid). *Nordaland***:** E6 road, contact with the Arctic Circle, 66º34'36"N, 15º20'57"E, 679 m, 14 August 2015, on soil with mosses and lichens, *C. G. Boluda & N. Calpena* (MAF-Lich. 20107, DNAcode 5079, TLC**:** norstictic acid).—**Portugal:** *Beira*

*Baixa***:** Covilha, Serra da Estrela, Torre, 40º19'N, 07º36'W, 1966 m, on granitic boulders, 11 June 2014, *V. J. Rico* (MAF-Lich. 19472, DNAcode 4590). *Minho***:** Peneda-Gerés Natural Park, *c*. Castro Laboreiro, 42º01'N, 08º08'W, 1028 m, 9 September 2014, *C. G. Boluda & V. J. Rico* (MAF-Lich. 19473, DNAcode 4670).—**Romania:** *Hunedoara***:** Vrdele Pass, 45º20'40"N, 23º39'31"E, 2115 m, 12 October 2014, on siliceous rock, *C. G. Boluda & E. Araujo* (MAF-Lich. 19475, as *P. pubescens*, DNAcode 4668).— **Spain:** *Asturias***:** Caso, Campo de Caso, Redes Natural Park, Valdebezón, from Monasterio river to peña'l Vientu, 43º05'21"N, 05º19'33"W, 1553 m, S slope, on quartzite, 6 September 2012, *V. J. Rico 4423* (MAF-Lich. 17838, as *P. pubescens*, DNAcode 4199). *Segovia***:** La Granja de San Ildefonso, *c.* Puerto de Navacerrada to Puerto de Cotos road, brook of Los Puentes, towards the Loma del Noruego, 40º47'36"N, 03º57'12"W, 1900 m, 7 March 2014, on granite, *V. J. Rico 4614* (MAF-Lich. 20103 (DNAcode 5071, TLC**:** norstictic acid; MAF-Lich. 20104, DNAcode 5072, TLC**:** norstictic acid).—**Sweden:** *Jämtland***:** Kall par., Skäckerfjällen Nature Reserve, Rutsdalen, SW slope of Dörsvalen, 63.80775ºN, 12.84044ºE, 700 m, on siliceous rock in boulder area, 15 August 2010, *M. Westberg 10-078* (S F177970, DNAcode 4367). *Lycksele Lappmark***:** Tärna sn, Atoklintens, 65º40.904'N, 14º38.310E, on rock, 14 August 2012, *G. Odelvik 12609*, *M. Hamnede & L. Hedenäs* (S F240229, DNAcode 4364).—**USA:** *Alaska***:** Fairbanks North Star Co., Steese Highway, Eagle Summit, 65.4867, -145.4153, 1100 m, alpine tundra, on rocks, 31 July 2011, *R. Rosentreter 17334*, *17292 et al.* (SRP L8791, L8806, as *P. pubescens*), DNAcode 4339, 4332). *California***:** Placer Co., Tahoe Rim Trail just north of Blockway Summit Trailhead, 39.2646ºN, 120.0607ºW, 2361 m, on granite, 9 February 2015, *N. Noell 1581 & J. Hollinger* (Hb. N. Noell, as *P. pubescens*, DNAcode 4781). *Montana***:** Deerlodge Co., Beaverhead-Deerlodge National Forest, c. Four Mile Basin, 46º05.680'N, 113º13.918'W, 2438 m, on granite boulders, 8 August 2009, *St. Clair 16685* [St. Clair *et al*. Lich. W. N. Amer. no. 145] (S F175892, as *P. pubescens*, DNAcode 4363); Jefferson Co., just E of Homestake Pass on I90, 45º55'01"N, 112º22'33"W, 6200 ft [1890 m], on boulders, 12 July 2006, *C. M. Wetmore 94730* (S F144171, as *P. pubescens*, DNAcode 4362). *Nevada***:** White Pine Co., Great Basin National Park, Mount Wheeler, 38.9859ºN, 114.3148ºW, 3981 m, rocky summit, on quartzite, 4 October 2014, *N. Noell* (1442) *& J. Hollinger* (Hb. N. Noell, DNAcode 4784). *Washington***:** Spokane Co., Turnbull National Wildlife Refuge, Kepple Lake overlook, 47.441ºN, 117.528ºW, 700 m, on top of basalt on forest floor, 1 February 2015, *N. Noell 1557, J. Allen & R. O'Quinn* (Hb. N. Noell, as *P. pubescens*, DNAcode 4783).

***Pseudephebe pubescens* (L.) M. Choisy**

Icon. Lich. Univ., ser. 2, **1:** *sine pag.* (1930).—*Lichen pubescens* L., *Sp. Pl*. **2:** 1155 (1753); type**:** Dillenius, *Hist. Musc.***:** pl. 13, fig. 9 (1742) (lectotype designated by Jørgensen *et al*. 1994**:** 343); *sine loc.* (LINN 1273.286—epitype designated by Jørgensen *et al*. 1994**:** 343); **Sweden:** *Jämtland***:** Undersåker sn, Välliste, topplatån, 1300 m VSV om Norra stugan, 63º16'07"N, 13º08'16.1"E, fjällhed, klippor, på stenblock, 22 July 2009, *G. Oldevik 9115* (S F149572—***reference specimen selected here***, DNAcode 4365; GenBank accession numbers**:** ITS KU647317, RPB1 KU668513, MCM7 KU668477).

(Figs. 2E−F & 3D−F)

Jørgensen *et al*. (1994**:** 343) pointed out that the specimen in the Linnean collections under the name *Lichen pubescens* (LINN 1273.286), which had been cited as lectotype by Hawksworth (1972**:** 235), was annotated in the handwriting of Linnaeus' son and not Linnaeus himself, something also pointed out by Howe (1912**:** 201). In the absence of evidence that the specimen was part of the original material studied by Linnaeus prior to publication of the name, it is not eligible as a lectotype and the Dillenian plate has to be used. Jørgensen *et al*. (1994**:** 343) consequently designated LINN 1273.286 as epitype. As there appears to be no mechanism to change an epitype once selected, without going through the formal conservation process, we have indicated a reference specimen (RefSpec) to serve as a proxy sequenced type to fix our application of the epithet *pubescens,* as for *minuscula* (see above).

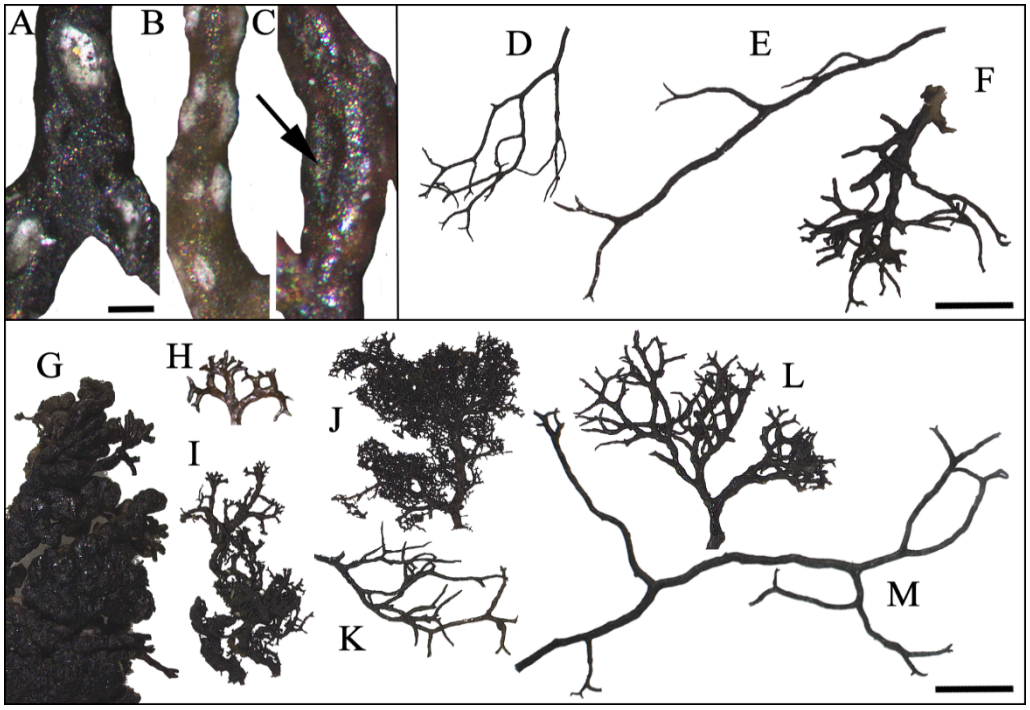

Fig. 3. Pseudocyphellae and branching variability in *Pseudephebe*. A–C, pseudocyphellae, *P. minuscula*; A, perforated (MAF-Lich. 20100); B, superficial (MAF-Lich. 19472); C, inconspicuous (MAF-Lich. 20103). D–F, *P. pubescens* branching variability; D, S F149572; E, MAF-Lich. 19474; F, MAF-Lich. 20101. G–M, *P. minuscula* branching variability; G, hb. N. Noell 1442; H, MAF-Lich. 20103; I, hb. N. Noell 1561; J, S F177970; K, MAF-Lich. 19475; L, MAF-Lich. 19472; M, NMW.C.2015.004.8. Scales**:** A–C = 0.1 mm; D–M = 2 mm.

We selected a sequenced reference specimen from northern Sweden, as Linnaeus (1753**:** 1155) gave the habitat information as "Habitat in Europa septentrionali, Lapponia, Suecia". Dillenius (1742**:** pl. 13 fig. 9) gives the locality as "In rupibus Groenlandie leóta & ab me delata fuit a Franc. Soldano, Chirurgo." (loc. cit.**:** 66), i.e. from Greenland where it was evidently collected by Francesco (or perhaps Franco) Soldano (fl. 1700–1740), a surgeon and friend of Dillenius known to have collected at several sites in Greenland as far as 81°N. Some specimens of Soldano are evidently still present in the Sherard herbarium in OXF (Clokie 1964). Specimens in that herbarium are not available for loan and have not been studied by us. Although potentially of historical interest, they are not now pertinent to the typification of the Linnean name as an epitype has already been designated by Jørgensen *et al*. (1994).

Of the samples analysed molecularly, specimens from Norway, Spain, Sweden, and Switzerland regions clustered in the *Pseudephebe pubescens* clade.

Table 3. Most discriminatory characters for the separation of *Pseudephebe* species, based on genetically analysed material. An unequivocal identification to species is only possible using DNA barcoding.

Character	*Pseudephebe minuscula*	*Pseudephebe pubescens*
Habit	Rarely subcrustose, small to large fruticose	Never subcrustose, small to medium fruticose
Thallus size (diameter)	Usually less than 3 cm, but reaching more than 8 cm	Less than 5 cm
Internode length	Usually less than 1 mm, but reaching 7 mm	Usually 1−3 mm, but sometimes less than 1 mm
Compressed old branches	Frequent	Rare
Flattened branching	Frequent	Rare, but never very flattened
Richly branched tips	Frequent	From absent to present in the same specimen
SSU-3' 1516 intron	Frequent	Absent

Specimens examined (DNA, morphology, and chemistry examined). **Norway:** *Sogn og Fjordane***:** Sogn, c. Luster, 61º31'58"N, 07º50'49"E, 1316 m, 12 August 2015, on rocky soil with mosses and lichens, *C. G. Boluda & N. Calpena* (MAF-Lich. 20100, 20102, DNAcode 5081, 5080, TLC**:** norstictic acid). *Nordaland***:** E6 road, contact with the Arctic Circle, 66º34'36"N, 15º20'57"E, 679 m, 14 August 2015, on soil with mosses and lichens, *C. G. Boluda & N. Calpena* (MAF-Lich. 20108, DNAcode 5078, TLC**:** *cf.* gyrophoric acid).—**Spain:** *Asturias***:** Caso, Campo de Caso, Redes Natural Park, surroundings of Ubales Lake, N slope of Cascayón pike, 43º06'05"N, 05º21'16"W, 1750 m, 8 September 2012, on quartzite, quartzite vertical wall, on sedges, other lichens and bryophytes, *V. J. Rico 4490, 4498, 4513* (MAF-Lich. 17907, 17915, 17930, DNAcode 4198 (TLC**:** norstictic acid), 4196, 4197); 43º06'09"N, 05º21'11"W, 1750 m, 8 September 2012, on quartzite, *C. G. Boluda 88* (MAF-Lich. 18112, DNAcode 3709). *León***:** Riaño, Picos de Europa, close to the village, 43º07'47"N, 04º58'59"W, 1 November 2014, on siliceous conglomerates, *C. G. Boluda & N. Calpena* (MAF-Lich. 19474, DNAcode 4671). *Teruel***:** Orihuela del Tremedal, Nuestra Señora del Tremedal Sanctuary, 30TXK143874, 1725 m, 4 September 2010, *Pinus sylvestris* forest, on quartzite, *V. J. Rico 4032 & M. Vivas* (MAF-Lich. 16841, DNAcode 4200). *Zamora:* Sanabria Lake, close to the Laguna de los Peces, 42º09'50"N, 06º44'10"W, 1641 m, 28 July 2013, rocky areas among bushes, *C. G. Boluda & N. Calpena* (MAF-Lich. 19470, 19471, DNAcode 4589, 3919).—**Switzerland:** *Uri***:** c. Wassen, 46º45'08"N, 08º29'01"'E, 2210 m, alpine siliceous rocky outcrops, 15 June 2014, *C. G. Boluda & Ch. Scheidegger* (MAF-Lich. 19476, DNAcode 4669).

Discussion

Pseudephebe is confirmed as a separate genus in the alectorioid clade of *Parmeliaceae*, with two genetically isolated clades, recognized here as *P. minuscula* and *P. pubescens*. Pseudocyphellae (Fig. 3), norstictic acid, and other extrolites are common characters in the genus, although these have not previously been recognized. *Pseudephebe pubescens* and *P. minuscula* are not morphologically always distinguishable, as indicated by annotations of specimens examined by numerous lichenologists, and our molecular data. The morphological variability of *P. pubescens* overlaps that of the more variable *P. minuscula*. Many specimens are distinguishable with confidence only by DNA analyses, especially of the ITS region, proving consequently to be cryptic. Nevertheless, specimens with extreme morphologies–subcrustose thalli, considerably flattened branching patterns or very compressed branches–as well as samples with an intron at the end of the SSU-3' locus, are most likely to belong to *P. minuscula*.

In some cases, as in the following Norwegian analysed samples, MAF-Lich. 20108, 20107, 20102, 20100 and 20101, *Pseudephebe minuscula* and *P. pubescens* grow together with comparable morphology, suggesting that the final thallus form is environmentally determined, particularly in *P. minuscula*. Comparable situations relating thallus morphology to

environmental factors have been described in a range of other lichens (Hawksworth 1973) but many of those examples have yet to be examined by molecular methods. The underlying mechanisms of this process are unknown, and perhaps could be elucidated by gene-expression studies in due course. The possibility that some extra-chromosomal "evo−devo" processes (Sultan 2015) are involved may also merit investigation when technically feasible.

SSU-3' 1516 introns are common in *Parmeliaceae* (Gutiérrez *et al*. 2007), but a variable presence was not previously known to occur within a single species, as specimens with and without an intron generally belong to different cryptic species (Molina *et al.* 2011a, b). In other lichen groups such as in *Physcia*, belonging to *Physciaceae* (*Caliciales*), similar introns in close-by regions (e.g., the LSU) are common and variable in a single species and even in the same specimen (Simon *et al.* 2005). PCR is a process in which the commonest or the most competitive locus allele can be fixed on the resulting sequence, masking the probability of detecting within-locus variability. Two of our samples contained intron presence and absence at the same time, as the SSU is a multi-copy locus, we cannot establish if this variability is among different regions of the branches, different fungal partners growing in a single thallus or inside a single nucleus. This could indicate that SSU-3' 1516 intron is an unstable element that can be deleted from the genome and regained thorough 'homing' (homing endonuclease) or reverse splicing mechanisms (Haugen *et al*. 2004, Reeb *et al*. 2007; Bhattacharya *et al*. 2005). This intron seems present as an ancestral character in Clade A and then secondarily lost independently in some clades, individuals, thallus regions, or even in some of the copies in a single fungal nucleus.

Table 4. Species delimitation prediction according to each method and marker. A, A' and B refers to clades named in Fig.1.

Method/Marker	ITS	MCM7	RPB1	Concatenated
ABGD	A, B	A, B	A, B	A, B
PTP	A, A', B	A, B	A, B	A, B
BP&P	−	−	−	A, B

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Supplementary Material

Supplementary Material 1

Additional specimens examined, morphology and chemistry studied, organized according to the original identifications.

As *Bryoria mariensis:* **Falkland Islands:** *West Falkland***:** Port Howard, feldmark and outcrops on summit ridge of Mt. María, UTM 21F UC 2078-2079, 2000-2150 ft [610-655 m], 28 January 1968, *H. A. Imshaug* (41393) *& R. C. Harris* (MSC 80871); Mt Maria, slope above Castle Rock, 51º37'S, 59º35'W, 500 ft. [150 m], on short dry grassland and *Empetrum,* 11 January 1992, *R. I. Lewis Smith 8506* (AAS, as "*Bryoria falklandica"* ad int.).

As *Pseudephebe minuscula* or its synonyms (*cf.* Hillman 1936, Lamb 1964, 1948, Hawksworth 1972, Brodo & Hawksworth 1977). **Antarctica:** *Livingston Island***:** Hurd Peninsula, moraine along the Reina Sofía glacier, 150 m, 14-February-1990, *L. G. Sancho* (MAF-Lich. 6824); Falen Bay, 80 m, on granite, 09-February-1991, on granite, *L. G. Sancho* (MAF-Lich. 4130); South Bay**:** False Burdick, 400 m, 26 January 1991, *L. G. Sancho* (MAF-Lich. 4129); 80 m, on granite, 25 February 1990, *L. G. Sancho* (MAF-Lich. 4131). *Antarctic Peninsula***:** W coast of Horseshoe Island, Marguerite Bay, 23 February 1965, *R. E. Longton* 1270 (BM 1089100, as *Alectoria minuscula*; TLC**:** norstictic acid).—**Canada:** *Yucon Territory:* Slopes above W shore of Kusawa Lake, 18 km S of Alaska Hwy (Yucon 1), on road to Kusawa Lake Campground, 60º36'14"N, 136º08'05"W, 700 m, exposed volcanic rock outcrops with soil communities on E-facing slope, on rock, 07 June 2011, *J. C. Lendemer* 29275 (MAF-Lich. 18338). —**Greenland:** *Angmagssalik area***:** Quigertieraq, 40 m, on stone in moraine, 18 July 1969, *F. J. A. Daniëls* (D525) *& J. G. Molenaar* (K(M) IMI 145918, as *A. minuscula*; TLC**:** no substances detected).

*Central-West Greenland***:** Qeqertaq, 70º00'N, 51º19'W, on gneissic rock, 31 July 2003, *E. S. Hansen* (MAF-Lich. 19401).—**Norway: *Hordaland*:** Hardangervida Natural Park, 23 July 1988, *L. G. Sancho & A. Belio* (MAF-Lich. 11313).—**Spain: *Huesca*:** Aristas de Brazato, 2550 m, July 1994, *L. G. Sancho & al.* (MAF-Lich. 6130). *Salamanca***:** Sierra de Béjar, Circo del Calvitero, 2100 m, 24 October 1980, *L. G. Sancho* (MAF-Lich. 11314). *Segovia***:** La Granja de San Ildefonso, *c.* Puerto de Navacerrada to Puerto de Cotos road, brook of Los Puentes, towards the Loma del Noruego, 40º47'36"N, 03º57'12"W, 1900 m, 7 March 2014, on granite, *V. J. Rico 4614* (MAF-Lich. 20109).—**USA: *Arizona*:** Coconino Co., Coconino National Forest, San Francisco Peaks, 3500 m, on basalt, 12 June 1998, *T. H. Nash III* 42036 [Anonymous, *Lich. Exs. Arizona State Univ.* no 329] (MAF-Lich. 6750). *Idaho***:** Lemhi Co., Salmon-Challis National Forest, Lemhi Mountains, Meadow Lake, 44.4324ºN, 113.3249ºW, 2869 m, on quartzite in alpine ridge, 4 June 2013, *N. Noell* (1561) *& J. Hollinger* (Hb. N. Noell).—**Switzerland: *Canton Valais*:** Zermatt, below Schwartzsee, 2450 m, 24 September 1972, *A. M. Burnet* 451 (BM s. n.; TLC**:** norstictic acid). *Uri***:** c. Wassen, 46º45'08"N, 08º29'01'"E, 2210 m, alpine siliceous rocky outcrops, 15-June-2014, *C. G. Boluda & Ch. Scheidegger* (MAF-Lich. 19477) —**USA: *Colorado*:** Boulder Co., West of Diamond Lake, 11600 ft. [3535 m], 21 August 1964, *S. Shushan* 4814 (BM 1089099, as *A. minuscula*; TLC**:** unidentified substance 1).

As *Pseudephebe pubescens* or its synonyms (*cf.* Hillman 1936; Lamb 1964, 1948; Hawksworth 1972; Brodo & Hawksworth 1977). **Antarctica: *Livingston Island*:** South Bay, Spanish Antarctic Base, 30 m, 16 February 1995, *L. G. Sancho* (MAF-Lich. 10194); El Peñón line, 120 m, saxicolous, January-1990, *L. G. Sancho* (MAF-Lich. 4132); Bayers Peninsula, 15 m, 31 January 1990, *L. G. Sancho* (MAF-Lich. 4133); 60 m, 27 January 1990, on moraine at the edge of the glacier, *L. G. Sancho* (MAF-Lich. 4134); Reina Sofía, 270 m, February-1990, *L. G. Sancho* (MAF-Lich. 4135); Roca Partida**:** 110 m, 10 February 1990, *L. G. Sancho* (MAF-Lich. 4136); False Burdick, 400 m, 26 January 1991, *L. G. Sancho* (MAF-Lich. 4137); Caleta Argentina, 90 m, 14 February 1990, *L. G. Sancho* (MAF-Lich. 6829); Barnard Peninsula, False Bay, 300 m, top of the mount, 28 July 1995, *L. G. Sancho & A. Pintado* (MAF-Lich. 6816); Falen Bay, 80 m, on granite, 09 February 1991, on granite, *L. G. Sancho* (MAF-Lich. 4130); 80 m, on granite, 25 February 1990, *L. G. Sancho* (MAF-Lich. 4131).—**Australia: *Australian Capital Territory*:** Brindabella Range, summit of Mt. Franklin, 35º29'S, 148º47'E, 1644 m, on metamorphic rock outcrops with sparse vegetation, 03 November 1999, *S. H. J. J. Louwhoff, M. C. Molina* (350, 385) *& J. A. Elix* (MAF-Lich. 9697, 9725). *Tasmania***:** Mt Field National Park, Mt Field West Plateau, 1400 m, on calcite boulders, 11 March 1980, *G. Kantvilas* 32/80 (BM 1089102; TLC**:** norstictic acid).—**Austria: *Tirol*:** Grieskogel und Larstigkopf, 2700 m, October 1975, *G. Follmann* [Follmann, *Lich. Exs. Sel. Cassel*. no. 237] (MAF-Lich. 1109).—**Chile: *Magallanes y Antártida Chilena (Región XII)*:** Navarino Island, Bandera Hill, N slope, 54º57'37"S, 67º37'57"W, 550 m, alpine soil, 09 January 2005, *J. Etayo* (22271)*, A. Gómez-Bolea & L. G. Sancho* (MAF-Lich. 15691, 15925); Puerto Williams, Virgen de Lourdes track to Barranca Guarriaco, 54º56'46"S, 67º34'52"W, 90 m, 14 January 2005, on soil, *J. Etayo* (22495)*, A. Gómez-Bolea, U. Søchting & R. Vilches* (MAF-Lich. 15828). —**France: *Corsica*:** Haute Corse, Corte, Gorges de la Restonica, a block of granite, 6 October 2011*, C. Gueidan* CG1982 (BM 980053; TLC**:** no substances detected).—**Greenland: *Angmagssalik area*:** SW Great Blomsterdalen, 80 m, 16 June 1969, *F. J. A. Daniëls* (D522) *& J. G. Molenaar* (K(M) IMI 145921, as *Alectoria pubescens*; TLC**:** norstictic acid). *South*

Greenland: Nanortalik, 60º09'N, 45º15'W, on gneissic gravel with *Alectoria sarmentosa* subsp. *vexillifera*, *Sphaerophorus globosus*, *Umbilicaria hyperborea* and *U. torrefacta*, 29 July 2004, *E. S. Hansen* (S L65904). *South West Greenland***:** Alluitsup Paa (Sydprøven), 60º28'N, 45º35'W, on stone together with *Parmelia saxatilis, Rhizocarpon geographicum* and *Umbilicaria hyperborea*, 31 July 2008, *E. S. Hansen* [*Hansen, Lich. Groenl. Exs.* no 1049] (S F169137).—**Ireland:** *Galway Co.***:** Benchoona, 1850 ft. [560 m], 27 June 1966, *D. L. Hawksworth* 506 (BM 1089094; TLC**:** no substances detected); near Leckavrea Mt., rocks near loch, 2000 ft. [610 m], 25 June 1966, *D. L. Hawksworth* 485 (BM 1089095; TLC**:** no substances detected).—**Japan:** *Hokkaido*, *Prov. Ishikari***:** [Kamikawa District] Mt. Daisetsu National Park, Mt Antaromadeke, 2200 m, 23 August 1971, *I. Yoshimura* 12425 [*Yoshimura, Lich. Japon. Exs.* no. 2, as *Alectoria pubescens*] (K(M) IMI 172381; TLC**:** norstictic acid).—**Norway:** *Lofoten Island***:** 100 m, 31 June 1988, *L. G. Sancho & A. Belio* (MAF-Lich. 1134).**—Spain:** *Asturias***:** Leitariegos, Laguna de Arvás, 06 September 1980, *A. Crespo & al.* (MAF-Lich. 1605). *Ávila***:** Sierra de Gredos, Circo de Gredos, 2040 m, 15 July 1982, *L. G. Sancho* (MAF-Lich. 11305). *Lérida***:** between Viella and Puerto de la Bonaigua, 2250 m, on rock, 22 July 1967, *H. Sipman* [*Stud. Biol. Rheno-Trai in itinere D 211*] (MAF-Lich. 982). *Logroño***:** Ezcaray, track to San Lorenzo Pike, 30TWM0276, 1780 m, screes, 07 September 2004, *A. Argüello* (MAF-Lich. 12522). *Madrid***:** Sierra de Guadarrama, 30TVL198233, 2340 m, 15 November 1988, *F. Valladares* (286) *& L. G. Sancho* (MAF-Lich. 13124); Cabezas de Hierro, 30TVL221172, 2240m, 21 November 1987, *F. Valladares* (288) *& L. Ramírez* (MAF-Lich. 11831); Cuerda Larga, 30TVL196166, 2150 m, 29 October 1988, *F. Valladares* 287 (MAF-Lich. 13125); Guarramillas, 30TVL180160, 2200 m, 21 November 1987, *F. Valladares* (288) *& L. Ramírez* (MAF-Lich. 13126); El Nevero, 30TVL302378, 2080 m, 15 January 1989, *F. Valladares* (290) *& L. Ramírez* (MAF-Lich. 13128); Cabeza Lijar, 30TVL019053, 1780 m, 01 November 1988, *F. Valladares* (291) *& L. Ramírez* (MAF-Lich. 13129); Valdemartín, 30TVL196166, 2150 m, 29 October 1988, *F. Valladares* 163 (MAF-Lich. 13345). Somosierra, 30TVL531545, 1750 m, 19 December 1988, *F. Valladares* 283 (MAF-Lich. 13121); 2000 m, *F. Valladares* 284 (MAF-Lich. 13122). Sierra de Camorritos, 30 June 1991, *A. Pintado* (MAF-Lich. 14204). Sierra de Abantos, 30TVL012043, 1780 m, 01 November 1988, *F. Valladares* 289 *& L. Ramírez* (MAF-Lich. 13127). Cercedilla, 30TVL090150, 1780 m, 16 December 1988, *F. Valladares* 285 (MAF-Lich. 13123). *Zaragoza***:** Moncayo, August-1898, on siliceous rocks (MAF-Lich.12764, as *Alectoria lanata*). *Zamora***:** Lago de Sanabria Natural Park, Lagunas del Padornelo, 29TPG7860, 1700 m, 09 September 1998, *A. Crespo* (MAF-Lic.6774); on *Erica* sp., *M. Pugh Jones* 1521 (MAF-Lich. 7206).**—Sweden:** *Åsele Lappmark***:** Vilhelmina sn**:** 65º06'N, 15º02'E, top of hill, on boulder, 01 July 2004, *G. Oldevik* 4508, 4315, 4307, 4304 (S L64662, L63058, L63068, L63070); 65º06'N, 14º27'E, top of hill, on *Betula nana* fallen death twig, 30 June 2004, *G. Odelvik* 4405, 4825 (S L63640, F57499); 65º07'N, 14º31'E, on pebbles on the ground, 30 June 2004, *G. Odelvik* 4401, 4527, 4850 (S L63644, L64642, F57452). *Hälsingland***:** Järvsö sn, top of hill, on boulder, 14 October 2005, *G. Oldevik* 5781 (S F52360). *Härjedalen***:** Tännäs sn, 62º20'24.2"N, 12º47'12.1E, sparse mixed forests, on rocks, 27 June 2007, *G. Oldevik* (7335) *& M. Myrdal* (S F79205); Vemdalen sn, Vemdalsskalet, Varggransfjället, on rocks, 03 July 2005, *G. Oldevik* 5424 (S L70225). *Pite Lappmark***:** Arjeplog sn, top of hill, on gravel, 22 August 2006, *G. Odelvik* 6399, 6108 (S F60992, F60239); on boulder with *Melanelia hepatizon*, 22 August 2006, *G. Odelvik* 6678 (S F192894). *Södermanland***:** Vårdinge sn, 59º05'08.2"N,

17º20’20.1”E, pine rocky ground, on cliff, 15 May 2012, *G. Odelvik* 12119 (S F235654).—**UK:** *S. Aberdeenshire***:** Braemar, summit of Morrone, [undated], *J. M. Crombie* [*Crombie, Lich. Brit. Exs.* no. 20, as *Alectoria lanata* var. *parmelioides*] (K(M) IMI 109522 also as *Pseudephebe pubescens*; TLC**:** norstictic acid, unidentified substance 2). *E. Inverness***:** Cairngorm Mountains, Cairn Gorm, 4000 ft. [1220 m], 2 August 1968, *D. L. Hawksworth* 1347 (BM 1089096; TLC**:** norstictic acid). *Shetland Islands:* Mainland, Ronas Hill, on granite boulders, 22 July 1966, *D. L. Hawksworth* 538 (BM 1089098; TLC**:** no substances detected). *Wales***:** Caernarvonshire, Cader Idris, 1877, *J. Carroll* (BM 1089097; TLC**:** no substances detected).—**Canada:** *Nunavut***:** Baffin Island, 31 km SE of Nanisivik, on granite stones, 13 July 1999, *P. K. Wong* 4679 (BM 1089101; TLC**:** norstictic acid).

Supplementary Material 2

Data on apothecial morphological characters in *Pseudephebe* studied specimens.

Specimens with apothecia**:** S-F149958, hb. N. Noell 1442 and MAF-Lich. 20102 in Clade A; MAF-Lich. 20100 in Clade B.

Ciliate apothecia were observed in both clades, and spore measurements were not discriminatory.

The mean values for 30 spores in the specimens with apothecia were:

- S F149958–8.93 × 5.92 µm, σ = 0.74 × 0.72 µm, Clade A
- hb. N. Noell 1442–8.12 × 5.96 µm, σ = 0.87 × 0.90 µm, Clade A
- MAF-Lich. 20102–8.50 × 6.80 µm, σ = 1.00 × 0.65 µm, Clade A
- MAF-Lich. 20100–9.30 × 5.90 µm, σ = 0.57 × 0.96 µm, Clade B.

We did not, however, compare length/width ratios, undertake further metric studies on the spores, or examine any pycnidia in view of the small numbers present in our material.

Supplementary Material 3

Node ages estimation

Materials and Methods

The molecular dating of tree-nodes was performed with BEAST v1.8.2 (*Drummond et al*. 2012) using a relaxed clock model (uncorrelated lognormal) with a birth-death model prior for the node heights and unlinked substitution models across the loci. The nucleotide-substitution model and parameters for all nucleotide-sites across each marker were selected using the Akaike information criterion (AIC) as implemented in jModelTest (Posada 2008). Mutation rates used were 2.43 x 10^{-9} $s \cdot s^{-1} \cdot yr^{-1}$ for ITS region, estimated from the parmelioid genus *Melanelixia* (Leavitt *et al*. 2012b), and 2.57 x 10^{-9} $s \cdot s^{-1} \cdot yr^{-1}$ for RPB1 and 1.73 x 10^{-9} $s \cdot s^{-1} \cdot yr^{-1}$ for MCM7 estimated from the family *Parmeliaceae* excluding the basal genus *Protoparmelia* (Amo de Paz *et al*. 2011; Divakar *et al*. 2015). The analyses were run with 50 million generations and parameter values were sampled every 1000th generation. We plotted the log-likelihood scores of sample points against generations using TRACER v 1.5 (Rambaut *et al*. 2014) and determined that stationarity had been achieved when the log-likelihood values of the sample points reached an equilibrium and ESS values exceeded 200 (Huelsenbeck & Ronquist 2001). In the absence of *Pseudephebe* fossils, a candidate alectorioid fossil (Kaasalainen *et al*. 2015) of 24-35 Mya was used as a calibration point for the group in a second run with the same parameters as above but with an estimation of the loci mutation rates.

Results

A calibrated maximum clade credibility chronogram analysis using predefined substitution rates is shown in Fig. S1. Analysis using a candidate alectorioid fossil as calibrator (Kaasalainen *et al*. 2015) and calculating the loci mutation rates, resulted in the next estimation**:** (a) 35.8 Mya (95 % HPD = 23.9 - 45.8 Mya) for the origin of *Pseudephebe*. (b) 11.6 Mya (95 % HPD = 5.5 - 18.5 Mya) for the split of clades A and B. (c) 4.2 Mya (95 % HPD = 2.4 - 6.3 Mya) for clade A diversification. (d) 1.1 Mya (95 % HPD = 0.5 - 2.0 Mya) for clade B diversification. (e) 42.5 Mya (95 % HPD = 37.3 - 49.8 Mya) for the origin of the Alectorioid clade. The fossil used as calibration point, however, could be an *Oropogon* and not an alectorioid species (Kaasalainen *et al*. 2015). The analysis using defined mutation rates (Fig. S1) suggest that *Pseudephebe* as a genus could have arisen c. 26.6 Mya in the Oligocene (95 % HPD = 20.6 - 31.8 Mya). In the absence of data from *Nodobryoria* species, this estimate needs to be treated with caution. If *Nodobryoria* proved to be a sister group to *Pseudephebe* (Divakar *et al*. 2015), a younger date might be supported. Divergence between the two

Pseudephebe species was estimated at 9.5 Mya (95 % HPD = 5.5 - 14.0 Mya), with 4.3 Mya (95 % HPD = 2.9 - 5.9 Mya) for the diversification of Clade A, and 1.2 Mya (95 % HPD = 0.6 - 2.2 Mya) for Clade B. As could be expected in Clade A, the high genetic variability resulted in older intraspecific clades. These results should be interpreted with caution, given the confusing fossil as well as the non-clock like topology of the phylogenetic tree in which they are based.

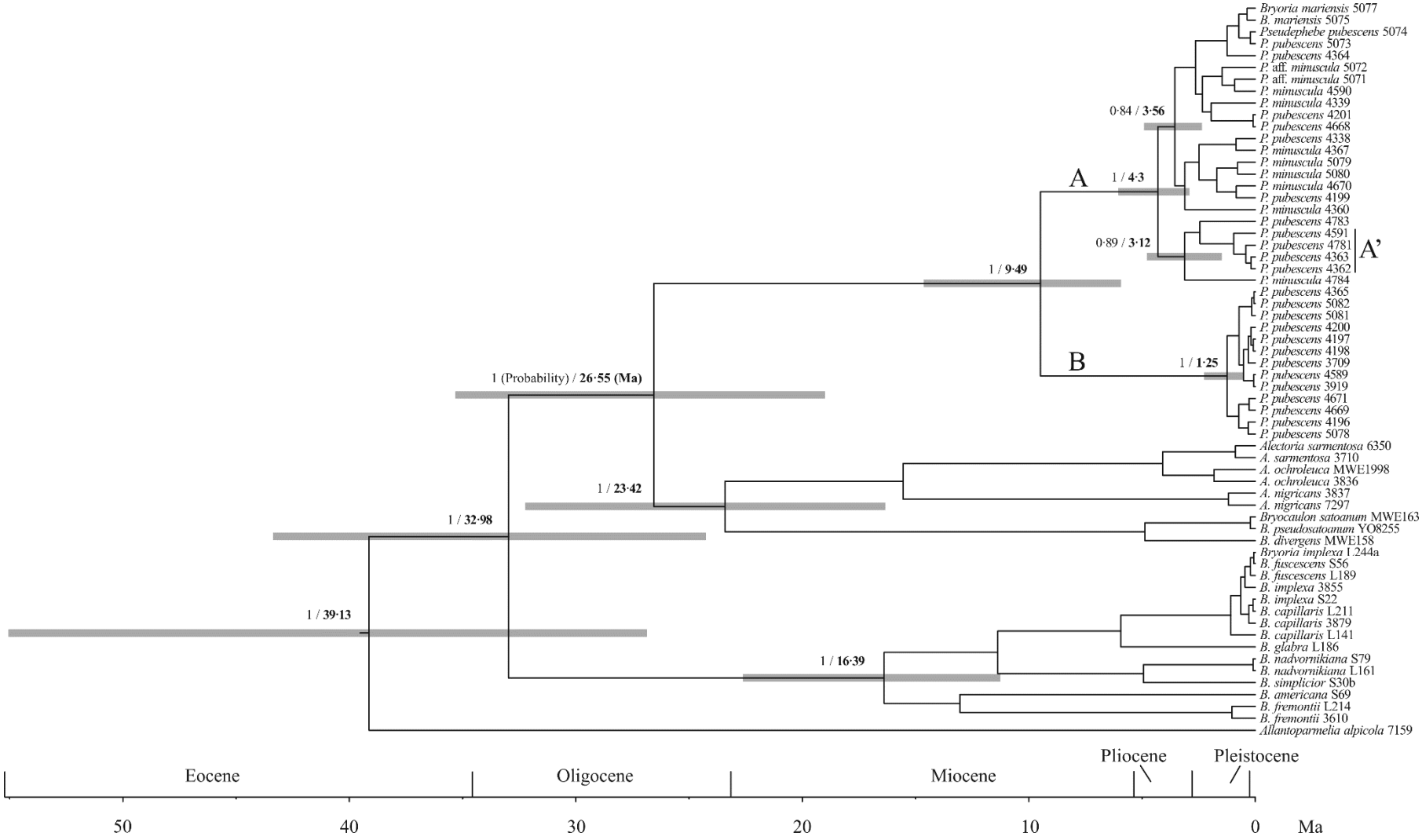

Fig. S1. Dated BEAST maximum clade credibility tree estimated from three-loci concatenated data. Grey bars indicating the 95% highest posterior density interval for the estimated divergence times. Posterior probabilities of interesting nodes and its divergence time as the mean posterior estimate of their age in Mya. Clades A, A' and B indicated as in Fig. 1.

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Discusión General

Bryoria fuscescens **creciendo favorecida por las actividades humanas. Noruega. (foto: C. G. Boluda).**

Discusión General

Cada capítulo cuenta con una discusión propia, a continuación, se discuten de forma sindicada los principales resultados obtenidos. Para facilitar la lectura de los taxones en la discusión, la siguiente tabla muestra las especies que incluye cada taxón, según se comenten desde un punto de vista morfológico o filogenético:

Concepto	Taxón	Especies incluídas
Concepto morfológico	*Bryoria fuscescens* s. str.	*B. fuscescens*
	B. fuscescens s. l.	*B. austromontana*, *B. chalybeiformis*, *B. friabilis*, *B. fuscescens*, *B. implexa*, *B. inactiva*, *B. kockiana*, *B. kuemmerleana*, *B. lanestris*, *B. pseudofuscescens*, *B. salazinica* y *B. vrangiana*
	B. sect. *Implexae*	*B. austromontana*, *B. capillaris*, *B. chalybeiformis*, *B. friabilis*, *B. fuscescens*, B. *glabra*, *B. implexa*, *B. inactiva*, *B. kockiana*, *B. kuemmerleana*, *B. lanestris*, *B. pikei*, *B. pseudofuscescens*, *B. salazinica* y *B. vrangiana*
Concepto filogenético	Fenotipo-*capillaris*	*Genepool* 1 de *B. fuscescens* s. str.
	Fenotipo-*fuscescens*	*Genepool* 2 de *B. fuscescens* s. str.
	B. fuscescens s. str.	*B. fuscescens*
	B. fuscescens agg.	*B. fuscescens*, *B. kockiana* y *B. pseudofuscescens*
	B. sect. *Implexae*	*B. fuscescens*, *B. glabra*, *B. kockiana* y *B. pseudofuscescens*

Los extrolitos en los líquenes alectorioides

Las sustancias liquénicas han sido muy utilizadas en los líquenes alectorioides, tanto como carácter clave para diferenciar especies, como de forma secundaria para complementar su descripción. Nuestros resultados remarcan, en primer lugar, la importancia de aplicar una metodología adaptada al organismo. En los líquenes alectoriodes, las concentraciones en extrolitos de los talos pueden ser muy bajas, especialmente cuando las rutas metabólicas

presentan muchos pasos intermedios en los que las sustancias quedan acumuladas durante cierto tiempo, este sería el caso de los grupos de sustancias quimiosindrómicas. Para resolver esta situación, hemos resuelto concentrar los extractos liquénicos que se analizan mediante la técnica de TLC, lo que permite detectar las sustancias minoritarias que de otra forma pasan desapercibidas. Esto pasa en *Bryoria* sect. *Implexae* con la atranorina y en *Pseudephebe* con el ácido norestíctico. Para ello, mantenemos porciones del talo en acetona a 50 ºC, dejándola evaporar parcialmente durante unos 10-15 min, de tal manera que los solutos quedan concentrados.

Consideramos que la composición particular en extrolitos de los taxones analizados en esta memoria, no debe de ser usada para caracterizar a las especie resultantes de los análisis moleculares filogenéticos. En *Pseudephebe*, la presencia de ácido norestíctico es altamente polifilética (Capítulo 7). En *Bryoria araucana*, las sustancias detectadas son las que con más frecuencia aparecen en el género (Capítulo 6). Finalmente, en *Bryoria* sect. *Implexae*, se detecta una amplia variedad de sustancias, independientemente del grado de parentesco genético entre los individuos (Capítulos 2, 4 y 5). Según nuestros análisis, algunos compuestos entendemos que están ligados a factores ecológicos, como los ácidos girofórico y psorómico (Capítulo 5). En *Bryoria fuscescens* s. str., los diferentes quimiótipos detectados, parecen estar fijándose en algunos grupos genéticos, como ocurre en el *Genepool 1*, que no produce ácido fumarprotocetrárico, pero con una tendencia a sintetizar ácido barbatólico (Capítulo 5).

Las sustancias liquénicas tienen importancia taxonómica en algunos líquenes alectorioides no estudiados en esta tesis, sin embargo, esta es mucho menor de la que en los estudios más clásicos se le había otorgado. Las sustancias químicas por si solas, no deben ser utilizadas para una identificación específica fiable, si no van ligadas a características morfológicas complementarias. Por lo tanto, la quimiotaxonomía en los líquenes alectorioides debería ser tenida en cuenta de forma menos destacada que hasta ahora.

La autofluorescencia, especialmente como metodología complementaria, ha resultado ser de mucha útilidad para localizar microscópicamente el lugar de los talos donde se acumulan los extrolitos. Sin embargo, está técnica no siempre permite distinguir con exactitud de que sustancias se trata. Por ejemplo, los ácidos fumarprotocetrárico, psoromico y norestíctico, son químicamente similares y producen fluorescencia con el mismo color azulado en las condiciones analizadas. Sin embargo, la atranorina o el ácido vulpínico, producen una coloración distinta, de tonos amarillos. En *Bryoria fremontii* la autofluorescencia permite localizar y distinguir el ácido vulpínico de otras sustancias minoritarias como el norestíctico. En otros parmeliáceos, como *Parmelia sulcata* (material suplementario del Capítulo 1), se

detecta la ubicación diferencial de la atranorina en el córtex y el ácido salizínico en la médula. No obstante, existen sustancias que parecen no producir autofluorescencia bajo las condiciones utilizadas, como el ácido úsnico (material suplementario del Capítulo 1). La autofluorescencia además permite estudiar la acumulación de otras sustancias, como las que aparecen en el epicórtex.

Datación de nodos

Conocer el tiempo de divergencia entre dos posibles especies es un carácter muy útil para determinar si merecen ser consideradas como diferentes. Esta es una de las principales razones por las que las edades de los nodos se han calculado en la presente tesis. Por ejemplo, en *Pseudephebe* (Capítulo 7), los dos grandes linajes obtenidos son crípticos, pero se estima que divergieron hace unos 9 millones de años, con lo cual, podemos confirmar que deben de considerarse especies distintas atendiendo a lo descrito en otros *Parmeliaceae* (Leavitt *et al*. 2012a; Divakar *et al*. 2015). En el caso de *Bryoria* sect. *Implexae* (Capítulo 5), los linajes son extraordinariamente recientes y no se pueden tomar decisiones basadas solo en este dato.

La metodología de datación de nodos se sustenta principalmente en dos tipos de datos, la tasa de mutación de las regiones de ADN y la presencia de fósiles que puedan ser utilizados como puntos de calibración. Esta metodología puede funcionar de manera aceptable en organismos muy bien estudiados y con un registro fósil abundante, como los vertebrados, sin embargo, esto no ocurre en los líquenes. Existen pocos trabajos previos en los que se hayan estimado las tasas de mutación de marcadores moleculares en líquenes, los cuales, además, utilizan como puntos de calibración los pocos fósiles existentes, algunos de ellos de dudosa interpretación (e .g. Amo de Paz *et al.* 2011; Leavitt *et al.* 2012; Divakar *et al.* 2015; Kaasalainen *et al.* 2015). Por estas razones, las tasas estimadas pueden contener sesgos, incluso suponiendo que el marcador en cuestión tenga una tasa de mutación más o menos constante a lo largo de todos los linajes testados.

Las investigaciones en las que se datan reconstrucciones filogenéticas, como las realizadas en los Capítulos 4 y 7, se valen de tasas de mutación previamente estimadas en otros estudios. Puesto que se utilizan marcadores neutros, se espera que estas tasas funcionen bajo el principio de reloj molecular y sean parecidas entre ellas independientemente del linaje. Sin embargo, la tasa de mutación puede variar dependiendo de algunas características propias de las especies, como el modo de reproducción, la duración de los ciclos de vida, frecuencia y duración de los periodos de actividad fisiológica, la presión de

selección o el tamaño poblacional. En nuestro caso, se han utilizado tasas de mutación de líquenes con ciclos de vida similares (Leavitt *et al.* 2012), aunque solo se dispone de datos de la región ITS, que ha mostrado ser poco variable en este grupo. Para minimizar estos errores se han realizado multitud de análisis testando en cada uno de ellos diferentes puntos de calibración, ya sean fósiles de dudosa ubicación (Kaasalainen *et al.* 2015) o edades de nodos previamente estimadas en otros estudios (Divakar *et al.* 2015). Todos estos problemas pueden ser tamponados durante el análisis eligiendo las metodologías más adecuadas para cada tipo de datos y topología del árbol.

Las dataciones aquí realizadas, al igual que en cualquier trabajo liquenológico, deben de ser tomadas con cautela. En cualquier caso, y aunque el margen de error pudiera ser muy elevado (pese a que las barras de 95 % HPD indican lo contrario), *Bryoria fuscescens* agg. incluye especies extraordinariamente recientes. Estas constituirían las más recientemente originadas de todos los líquenes hast ahora estudiados, y son comparables con algunos de los procesos de especiación más recientes que se conocen, como el caso de los pinzones de las Galápagos (Lamichhaney *et al.* 2015) o especies de origen híbdrido del género *Linaria* (Blanco-Pastor *et al.* 2012).

Delimitación de especies

Son numerosos los programas estadísticos para delimitar especies, basados en diversos tipos de aproximaciones metodológicas. Podríamos pensar que estos programas permiten objetivizar la delimitación de especies, sin embargo, en la práctica y con frecuencia muestran resultados contradictorios cuando son aplicados en grupos filogenéticamente complejos (Alors *et al.* 2016; Del-Prado *et al.* 2016). Tanto en el Capítulo 4 como en el 7, podemos comprobar que las distintas metodologías, pese a utilizar los mismos datos, predicen un número variable de especies. Algunos programas, como ABGD, utilizan distancias genéticas obtenidas a partir de marcadores de ADN estándar para detectar brechas entre linajes. Sin embargo, la significatividad de las barreras detectadas parece dependiente del grado de diferenciación del grupo. Por ejemplo, el leve aislamiento de las especies de *Bryoria fuscescens* agg. quedaría prácticamente reducido a cero si otras especies del género, filogenéticamente más aisladas, se incorporaran al análisis. Además, las barreras pueden llegar a representar un artefacto, producido por un tamaño muestral no adecuado o debido a la ausencia de linajes no muestreados. Las metodologías basadas en coalescencia están cobrando cada vez más relevancia en la delimitación de especies. GMYC incorpora las teorías de coalescencia en sus análisis, sin embargo, parece sobreestimar el número de especies,

no solo en nuestro caso, sino también en otros estudios (Alors *et al.* 2016; Del-Prado *et al.* 2016).

La experiencia aquí obtenida nos hace pensar que los programas de delimitación de especies, cuando son utilizados en complejos de especies poco diferenciadas, dan resultados variables. En estos casos, por sí mismo, ningún programa parece dar un resultado fiable, por lo que deberían de utilizarse conjuntamente para detectar una tendencia de predicción. De todas formas, las posibles predicciones de especies, obtenidas con estos análisis, deberían de ser complementadas con evidencias derivadas de otros datos independientes (árbitros). Estos programas detectan, simplemente, linajes que podrían ser considerados especies, pero es el investigador, con su experiencia en el grupo, el que debe de corroborar si merece la pena que sean reconocidos como tales.

En el caso de *Pseudephebe* (Capítulo 7), los análisis coinciden en reconocer dos taxones específicos, sin embargo, cuando se utiliza solo el marcador ITS, muy variable en este grupo, el programa ABGD detecta el clado más basal, incluido en lo que reconocemos como *P. minuscula*, como una especie distinta. Este resultado podría parecer bastante lógico, ya que se trata de un linaje relativamente antiguo y molecularmente diferenciado. Sin embargo, otros linajes no apoyados establecen una comunicación entre este clado y el resto en *Pseudephebe minuscula*, por lo cual carece de sentido considerarlo como un taxón distinto.

En el caso de *Bryoria fuscescens* agg. (Capítulo 5), tanto los análisis de detección de especies realizados con secuencias, como los realizados con microsatélites, coinciden en definir tres grandes grupos que aquí se han considerado como tres especies. Sin embargo y pese a la gran similitud genética entre todos los individuos, algunos análisis estiman hasta 5 especies. Se reflexionó la posibilidad de considerar a todo el agregado como una única especie con tres linajes en proceso de especiación, que podrían haber sido referidos, por ejemplo, como subespecies. Sin embargo, esta conclusión parecía contradecir los resultados obtenidos por los programas de delimitación de especies. Además, existe una tendencia actual a situar el rango de especie en la familia *Parmeliaceae* a unos niveles de diferenciación genética muy bajos (Molina *et al.* 2011a; 2011b; Altermann *et al.* 2014; Saag *et al.* 2014; Del-Prado *et al.* 2016). La utilización del rango de subespecie, que podría haber sido útil en este caso, no es habitual en liquenología, principalmente debido a que su utilización, con anterioridad, se basaba en el uso exclusivo de ciertos caracteres fenotípicos que no reflejaban diferenciación filogenética. Sin embargo, actualmente, podría resurgir este concepto desde un punto de vista filogenético (*cf.* Magain *et al.* 2016), en tal caso, los linajes aquí detectados podrían encajar mejor con este rango taxonómico que con el de especie.

Los análisis poblacionales, con el uso de los microsatélites, muestran la tendencia evolutiva a separar lo que denominamos fenotipo-*capillaris* y fenotipo-*fuscescens* en grupos genéticos, sin embargo, estos linajes están muy poco diferenciados tanto molecular como morfológicamente. Tal vez, para estos grupos, se podría aplicar el concepto de subespecie dentro de la especie *Bryoria fuscescens* s. str. En cualquier caso, la adjudicación de una categoría taxonómica para estos fenotipos es muy arriesgada sin un estudio que incluya poblaciones del total del área de distribución del taxón.

Distribución potencial

Los programas de predicción de la distribución potencial, han mostrado ser de gran utilidad y precisión para muchas especies (Williams *et al.* 2009; Barros *et al.* 2012). Los análisis caracterizan de forma bastante adecuada las preferencias climáticas para el género *Bryoria* (Capítulo 5), no obstante, descartan como zonas adecuadas las áreas de baja altitud del Norte de Europa, a pesar de tratarse de taxones con citas frecuentes en estas áreas. Debido a que sin un análisis molecular no es posible identificar las especies de *Bryoria* sect. *Implexae*, se ha decidido no utilizar ejemplares de herbario para el análisis. Esto limita considerablemente los datos bioclimáticos que el programa utiliza para caracterizar a este grupo, ya que disponemos de “solo” 64 puntos de muestreo. Nuestro muestreo no ha sido muy extenso en zonas boreales continentales, lo cual puede llevar a que este hábitat sea considerado por el análisis como poco adecuado. Además, puede que micronichos producidos por el bosque estén favoreciendo la presencia de especies de *Bryoria*, indetectables mediante este tipo de análisis. Por otro lado, en algunas de las áreas donde se detectan condiciones muy favorables para su desarrollo, no aparecen especies de *Bryoria*, como puede ser gran parte de Reino Unido, Irlanda o las montañas del Líbano. Con mucha probabilidad, esto parece que se debe a la intensa actividad humana desde tiempos histórico.

Los trabajos que hemos realizado en el campo, nos han hecho ver que al menos en el Centro y Sur de Europa, fenotipo-*capillaris* requiere de zonas más húmedas y prístinas que fenotipo-*fuscescens*. Con frecuencia, estas zonas presentan una mayor altitud sobre el nivel del mar. Esto se ve reflejado en la distribución potencial de ambos grupos, que pese a seguir el mismo patrón, este es levemente más restringido en el primer grupo, pero casi inapreciable a la resolución en la que se muestran aquí los mapas.

La predicción de la distribución en el pasado proporciona un gran potencial para detectar refugios en épocas glaciales e inferir patrones migratorios. No obstante, en nuestro caso, no apoya la hipótesis de refugios glaciares en el norte de Europa, los cuales parecen

haber existido tanto según nuestros datos como los de otros autores (Tzedakis *et al.* 2013). Esto podría ser debido a que las condiciones climáticas que permiten la existencia de estos refugios no han quedado representadas en los mapas bioclimáticos utilizados, así como tampoco lo estarían los nunataks en los que especies de *Bryoria* podrían haber sobrevivido.

La realización de estos análisis puede mejorar considerablemente el diseño de los muestreos en los estudios poblacionales, ya que permite detectar áreas en las que el organismo tiene alta probabilidad de aparecer, independientemente de si previamente han sido muestreadas. En nuestro caso, áreas de Turquía, Líbano, el Cáucaso, los Balcanes o Etiopía, podrían albergar grandes poblaciones de estos líquenes, siempre y cuando la actividad humana no haya sido acusada. Finalmente, la distribución potencial, pese a su margen de error, puede ser de gran importancia a la hora de mejorar las medidas de protección de especies amenazadas, así como estimar su posible reacción frente al calentamiento global.

Dispersión en *Bryoria fuscescens* agg.

Los trabajos poblacionales en líquenes, con frecuencia coinciden en mostrar haplotipos ampliamente distribuidos a nivel continental o incluso mundial (Printzen *et al.* 2003; Fernández-Mendoza *et al.* 2011; Widmer *et al.* 2012). A simple vista, esto parece indicar una gran capacidad de dispersión de los organismos. La aparente capacidad para cruzar barreras tan gigantescas como los océanos en las especies pantropicales o los trópicos en las polares, frecuentemente se adjudica al fenómeno de la zoocoria, generalmente mediada por aves. Existen casos de migración bipolar en plantas a través de aves que comen sus frutos (Brochmann *et al.* 2003), sin embargo, ningún trabajo ha mostrado su efectividad en líquenes. Aunque la zoocoria sea un fenómeno factible en *Bryoria*, la probabilidad de que un ave migratoria lleve propágulos en su cuerpo y estos encuentren un hábitat adecuado para crecer, debe de ser muy baja, e incompatible con los altos niveles de migración detectados en el Capítulo 5. Mientras que el *Genepool 2* incluye individuos que producen propágulos de reducido tamaño, como los soredios, el *Genepool 1* parece reproducirse solamente por fragmentación, lo que dificultaría todavía más la dispersión mediante aves. Por otro lado, los análisis de aislamiento por distancia detectan unas capacidades similares en ambos *genepools*, descartando que los grupos sorediados tengan una mayor eficacia dispersiva.

Nuestros resultados, tanto de secuencias como de microsatélites, parecen indicar altos niveles de haplotipos ancestrales compartidos entre clados, linajes y áreas geográficas. Los haplotipos compartidos entre regiones geográficas serían detectados por los análisis como

eventos de migración o señales de conectividad entre regiones actualmente aisladas. Estos haplotipos podrían haber surgido en épocas en las que las poblaciones estaban conectadas o vivían en refugios glaciales a partir de los cuales se colonizarían grandes áreas de Europa. La hipótesis de haplotipos ancestrales compartidos es congruente con la deriva génica y reparto incompleto de linajes que este taxón parece poseer. Además, trabajos poblacionales previos, realizados en especies de líquenes cosmopolitas de la misma familia, coinciden en concluir que esta hipótesis parece más probable que una alta frecuencia de dispersión a grandes distancias (Printzen *et al.* 2003; Fernández-Mendoza *et al.* 2011).

Los programas bioinformáticos que nos permiten detectar migración o aislamiento por distancia parecen funcionar bien en organismos diploides, sexuales y susceptibles a barreras geográficas, como los vertebrados. Sin embargo, en líquenes, los resultados parecen estar muy influenciados por la reproducción clonal y su naturaleza haploide. En cualquier caso, *Bryoria fuscescens* en sentido estricto, pese a ser una especie de origen muy reciente, se ha detectado en lugares tan dispares como Norteamérica, Europa, Asia y el Centro de África, por lo que sus capacidades de dispersión deben de ser notables. Hasta la fecha solo el clado sorediado (*Genepool* 2) de este taxón ha sido herborizado fuera de la zona de nuestro estudio, lo que podría sugerir la importancia de la dispersión mediante soredios.

Tendencias evolutivas en *Bryoria fuscescens* agg.

Los autores que han realizado estudios previos en *Bryoria* sect. *Implexae*, pese a concluir que las morfoespecies no son distinguibles filogenéticamente con los marcadores utilizados, han decidido no sinonimizarlas (Myllys *et al.* 2011). Esto es debido a que las morfoespecies parecen constituir entidades fenotípicamente definidas y pueden crecer juntas, en contacto físico en el mismo hábitat, por lo que deben de ser resultado de una diferenciación genética y no de una adaptación ambiental (Velmala *et al.* 2014).

Se han utilizado numerosos marcadores moleculares durante la realización de esta tesis, algunos de ellos de uso estándar en líquenes (ITS, IGS, GAPDH, nuLSU, mtSSU y MCM7) mientras que otros han sido específicamente diseñados para *Bryoria fuscescens* agg. (FRBi13, FRBi15, FRBi16, FRBi18, FRBi19), incluyendo además 18 marcadores de microsatélites. Las reconstrucciones filogenéticas pretenden perseguir la historia evolutiva de las especies, sin embargo, solo reconstruyen la historia de los marcadores, por lo que la elección de los marcadores adecuados es muy importante en taxones filogenéticamente complejos.

Los marcadores estándar parecen reflejar bien las relaciones evolutivas en los líquenes alectorioides, con la excepción de *Bryoria* sect. *Implexae*. En este grupo, las secuencias de ADN estándar han resultado ser muy poco variables. De entre estas, ITS, IGS y GAPDH son las más variables, sin embargo, prácticamente todo el poder de resolución recae en GAPDH. Las topologías producidas por estos tres marcadores son congruentes entre sí y llevan a la formación de un árbol filogenético aparentemente más relacionado con la corología que con los fenotipos (Capítulo 4). Los cinco loci obtenidos a partir de las regiones flanqueantes de los microsatélites son altamente variables, sin embargo, cada marcador proporciona una topología particular, incongruente con las otras y con los caracteres corológicos y fenotípicos (Capítulo 5). Esto puede deberse al fenómeno de reparto incompleto de linajes, como se espera de regiones intergénicas variables en organismos estrechamente emparentados entre sí. Sería de esperar que una reconstrucción filogenética que utiliza las 18 regiones flanqueantes de los microsatélites, se autocorrigiera a si misma tendiendo a esbozar los grupos revelados por los microsatélites. Esta tendencia ya se observa en las topologías de cada marcador de nuestro estudio, que tienden a separar en mayor o menor grado los *Genepool* mostrados por los microsatélites (Capítulo 5).

Los microsatélites, debido a su alta variabilidad, son capaces de mostrarnos procesos evolutivos muy recientes, incluso si los marcadores basados en secuencias están influenciados por el reparto incompleto de linajes (Jarne & Lagoda 1996). Los microsatélites, por una parte, reflejan aspectos corológicos congruentes con las secuencias, como la diferenciación de linajes entre Europa y América, pero, además, han mostrado capacidad para detectar linajes genéticos congruentes con datos fenotípicos. En cualquier caso, los *pools* genéticos mostrados por los microsatélites no coinciden exactamente con ninguna morfoespecie descrita, ya que dan más importancia a caracteres que previamente se pensaba que no eran relevantes, como la ausencia de ácido fumarprotocetrárico y de soralios. Sin embargo, de forma secundaria, se ve una tendencia a incluir los fenotipo-*capillaris* en un *pool* genético y los fenotipo-*fuscescens* en el otro (Capítulo 5).

Tanto los microsatélites como las secuencias apuntan a que el linaje europeo (*Bryoria fuscescens*) y el americano (*B. pseudofuscescens*) no están totalmente aislados, ni corológica ni genéticamente. Además, algunos individuos poseen características intermedias entre ambos, con secuencias clonales de especímenes de un grupo y *pools* genéticos que lo ubican en otro.

Una visión global del uso de todos estos marcadores parece sugerir que *Bryoria fuscescens* agg. es un linaje recientemente originado, con altos niveles de reparto incompleto de linajes, que parece acumular cambios genéticos y fenotípicos bajo un proceso de deriva

con bajos niveles de selección natural. Sin embargo, esta deriva, debido a barreras geográficas y leves adaptaciones fenotípicas, parece llevar a la formación de linajes corológicos y fenotípicos. *Bryoria glabra* es una especie bien delimitada, mientras que dentro de *B. fuscescens* agg. aparecen dos especies principalmente americanas (*B. pseudofuscescens* y *B. kockiana*) y una de amplia distribución (*B. fuscescens*) con posibles niveles de reparto incompleto de linajes entre ellas. Además, en Europa, *Bryoria fuscescens* parece sumida en un proceso evolutivo que tiende a formar dos grupos fenotípicos que más o menos encajan con los conceptos tradicionales de *Bryoria capillaris* (denominado aquí forma o morfoespecie *capillaris*) y *B. fuscescens* (denominado aquí forma o morfoespecie *fuscescens*), aunque con gran cantidad de excepciones.

Significado de la variación intraespecífica

Los líquenes alectorioides son morfológicamente simples, por lo que se espera que la variabilidad fenotípica de una especie se solape con la de otras. En el caso de *Bryoria araucana* (Capítulo 6), tanto sus caracteres químicos como los morfológicos, son los comunes al género, por lo que existen numerosas especies con una apariencia similar, las cuales en ocasiones pueden ser muy difíciles de distinguir (e. g. *Bryoria fuscescens*, *B. pseudofuscescens*, *B. glabra*, *B. trichodes, B. irwinii* o *B. alaskana*). *Bryoria araucana* parece tener una morfología estándar en el género, bastante bien adaptada a las condiciones ambientales en las que típicamente crecen las especies de *Bryoria*.

En el caso de *Pseudephebe* (Capítulo 7) la plasticidad fenotípica parece estar influenciada por el ambiente. Por ejemplo, cuando *Pseudephebe minuscula* crece en altas cumbres ventosas y frías, adquiere un hábito subcrustáceo, mientras que cuando vive en un ambiente como el de las islas subantárticas con influencia oceánica, más idóneo (Fryday *et al.* 2012), adquiere un porte arbustivo que ha llegado a confundirse con el de especies del género *Bryoria*.

En el caso de *Bryoria fuscescens* agg. (Capítulo 5), parece más difícil justificar la gran variabilidad fenotípica observada. Algunos caracteres, como la producción de ciertos extrolitos, la coloración o la cantidad de pseudocifelas por talo, parecen estar, al menos en parte, condicionados por factores ambientales. Otros, sin embargo, aparentan estar ligados a la genética, ya que aparecen en ejemplares que crecen en un mismo micronicho. No obstante, los caracteres fenotípicos, no estan fijados en los clados revelados por las secuencias de ADN, aunque se observa una tendencia en los grupos definidos por los microsatélites. Los resultados obtenidos en el Capítulo 5 indican que estos linajes parecen estar influenciados

por la deriva génica. En tal caso, los ejemplares irían acumulando mutaciones, lo que, en ausencia de una presión de selección, con el tiempo, conllevaría a formar un grupo fenotípicamente muy variable, en el cual la variabilidad no estaría relacionada con los clados. Parece ser que, en líquenes, la aparición de mutaciones fenotípicas no conlleva a tanta presión de selección como en organismos más complejos, como los vertebrados, en los que cualquier variación fenotípica suele conducir a un fallo fatal en el organismo o un empeoramiento en su interacción con el ambiente. Dicho de otra forma, es lógico pensar que la presión de selección pueda ser mayor en vertebrados que en líquenes.

Si surgen nuevas características y estas no provocan la muerte de los ejemplares (son neutrales), permanecerán en la población e irán evolucionando por deriva. Factores como el tamaño poblacional y el éxito reproductivo irán aumentando o disminuyendo la proporción de estos individuos. Con el tiempo, cuando la morfología derive demasiado, se puede esperar que la selección la perfile formando especies fenotípica y genéticamente definidas.

Perspectivas futuras

Como suele ocurrir en cualquier trabajo científico, intentar resolver una pregunta genera respuestas, pero también muchas preguntas nuevas. Durante el desarrollo de la presente tésis, se han obtenido resultados que permiten responder a los objetivos planteados. Pero, además, quedan hipótesis que se verían beneficiadas de análisis adicionales y questiones en las se podrían basar futuras investigaciones.

En el Capítulo 1 se utiliza la autofluorescencia para la identificación y localización, a nivel microscópico, de sustancias acumuladas en el talo liquénico. Esta es una metodología en la que se ha profundizado poco durante el desarrollo de la tésis, pero puede tener un potencial todavía por descubrir. Nuevos estudios utilizando diferentes longitudes de onda, sustancias puras y evaluando como la coloración emitida es influenciada por la que emiten sustancias colindantes, podría mejorar la detección e identificación de los extrolitos *in situ*.

En el Capítulo 3, se realiza una secuenciación masiva de varios ejemplares de *Bryoria* sect. *Implexae* para el diseño de marcadores de microsatélites. Esto ha generado una infinidad de secuencias de ADN que fueron almacenadas, pero que se podrían utilizar para realizar nuevos estudios. Por ejemplo, podrían permitir el diseño de marcadores de microsatélites para el fotobionte o detectar la presencia de genes fisiológicamente importantes, como los PKS (relacionados con la producción de extrolitos) o los genes MAT (relacionados con la reproducción sexual). Una búsqueda rápida de los genes MAT entre estas secuencias ha

mostrado que al menos están presentes sus dominios funcionales. Una búsqueda más exaustiva podría permitir diseñar primers específicos para estudiar la evolución de estos genes a lo largo de los procesos de especiación y su variabilidad en las distintas regiones geográficas.

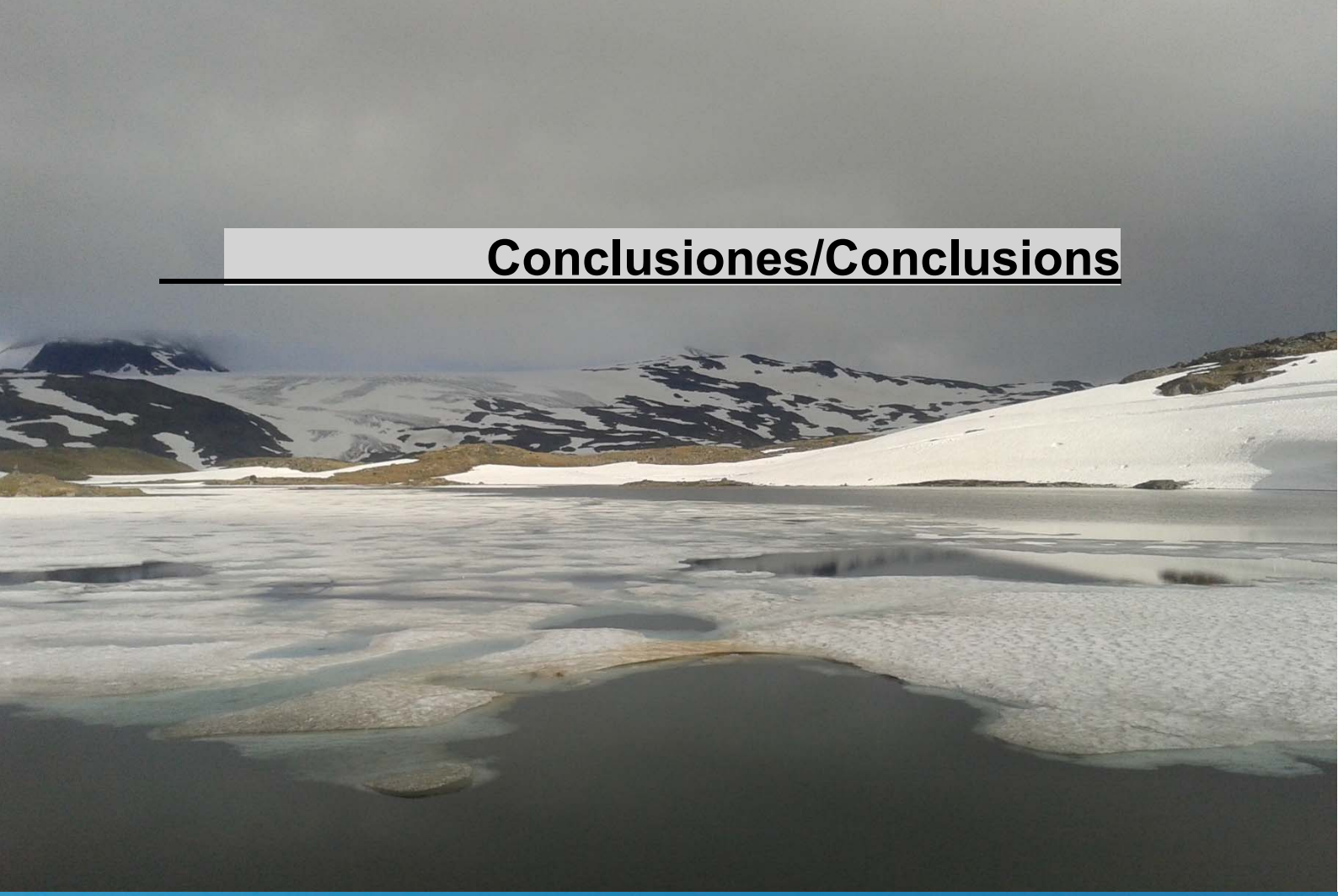
En el Capítulo 4, queda patente que un muestreo a nivel mundial es necesario para establecer un concepto de especie sólido en *Bryoria* sect. *Implexae*, ya que podrían aparecer nuevos linajes que conecten las especies aquí establecidas.

El Capítulo 5 es el que más hipótesis ha generado, para las cuales, análisis y muestreos adicionales podrían proporcionar respuestas. Previamente a la publicación de estos resultados, se pretenden realizar análisis adicionales, como los basados en alelos geográficamente restringidos (Widmer *et al.* 2012; Alors *et al.* 2017), que podrían mejorar la interpretación de los patrones geográficos o los flujos migratorios detectados. No cabe duda que un muestreo adicional y más amplio sería necesario para desvelar algunas de las hipótesis planteadas, como una posible participación de poblaciones asiáticas en la recolonización de Europa o el grado de conectividad entre Europa y América.

Respecto al Capítulo 6, los datos parecen sugerir que un muestreo exaustivo en Sudamérica desvelaría nuevas especies de líquenes alectorioides, de la misma forma que actualmente está ocurriendo en Norteamérica o Asia Central (Myllys *et al.* 2016; Wang *et al.* 2017).

En cuanto al Capítulo 7, un análisis que incorpore un mayor muestreo podría desvelar que *Pseudephebe pubescens* también aparece fuera de Europa. Además, el uso de más marcadores genéticos, podría desvelar si *Pseudephebe minsucula* es una única especie o un complejo de especies similar al detectado en otros parmeliáceos (Molina *et al.* 2011a; 2011b; Del-Prado *et al.* 2016).

Conclusiones/Conclusions



Círculo polar ártico (Noruega) y zonas alpinas tropicales (Kilimanjaro, Tanzania), dos ambientes en los que se ha recolectado *Bryoria fuscescens* (foto: C. G. Boluda).

Conclusiones

1. La fluorescencia se confirma como una herramienta útil para ubicar y en ocasiones identificar metabolitos secundarios acumulados en los talos liquénicos.

2. En *Bryoria fuscescens* agg., tanto la presencia como la composición en extrolitos puede ser variable en distintas regiones del talo y en ocasiones aparecer asociada a pseudocifelas o soralios.

3. La presencia y composición en extrolitos en *B. fuscescens* s. l. no están relacionadas con el parentesco genético y ni con las morfoespecies.

4. Las poblaciones de *Bryoria fuscescens* s. l. en la Región Mediterránea, muestran combinaciones de caracteres que no encajan con el concepto de especie establecido en poblaciones boreales.

5. Se han desarrollado marcadores de microsatélites específicos de *Bryoria* sect. *Implexae* que reúnen las características necesarias para ser utilizados en estudios poblacionales.

6. La taxonomía integrativa permite establecer un concepto de especie en *Bryoria* sect. *Implexae* que no revelan taxonomías basadas en solo un tipo de datos. De las 14 morfoespecies analizadas en esta tesis, solo cuatro se mantienen como especies, siendo *B. fuscescens*, *B. kockiana* y *B. pseudofuscescens* crípticas y *B. glabra* sutilmente distinguible.

7. Las especies de *Bryoria fuscescens* agg. constituyen el evento de especiación más reciente conocido en líquenes.

8. La especie *Bryoria fuscescens* s. str. presenta tres grandes grupos genéticos en Europa y Norte de África, dos de ellos de amplia distribución y uno restringido al norte de Escandinavia. Los rasgos genéticos de este último son intermedios entre *Bryoria fuscescens* y *B. pseudofuscescens*.

9. La elevada capacidad de dispersión detectada en *Bryoria fuscescens* s. str. parece ser el resultado de un artefacto producido por haplotipos ancestrales de amplia distribución.

10. La Península Escandinava, seguida por los Alpes y la Península Ibérica, constituyen las zonas con mayor riqueza genética de *Bryoria fuscescens* s. str. en Europa. La

diversidad genética de las poblaciones es independiente del desarrollo de apotecios en sus talos.

11. *Bryoria fuscescens* s. str. parece estar sumida en un proceso evolutivo de deriva hacia dos grupos fenotípicos con altos niveles de reparto incompleto de linajes.

12. El hongo liquenícola *Raesaenenia huuskonenii* puede crecer sobre *Bryoria* fuscescens agg. independientemente de la morfoespecie, quimiotipo o grupo genético.

13. Los especímenes de *Bryoria* recolectados en Chile se confirman como una especie nueva propuesta como *Bryoria araucana*.

14. *Bryoria mariensis* debe de considerarse sinónimo de *Pseudephebe minuscula*.

15. *Pseudephebe minuscula* es una especie muy variable cuya morfología se solapa con la de *P. pubescens*, por lo que ambas especies deben de considerarse crípticas.

Conclusions

1. The fluorescence is confirmed as a useful tool to locate and sometimes identify the secondary metabolites accumulated in the lichen thalli.

2. In *Bryoria fuscescens* agg., the presence and composition of extrolites can be variable among different thallus regions and sometimes can appear linked to pseudocyphellae or soralia.

3. The presence and composition of extrolites in *B. fuscescens* s. l. is neither related with genetic affinity nor morphospecies.

4. The populations of *Bryoria fuscescens* s. l. in the Mediterranean region show combination of characters that does not fit with the established morphospecies concept using boreal specimens.

5. New microsatellite markers specific for *Bryoria* sect. *Implexae* have been designed, containing the necessary requisites to perform phylogeographical studies at population level.

6. Integrative taxonomy allows to develop a species concept in *Bryoria* sect. *Implexae* that cannot be obtained using a single approach. Of the 14 morphospecies analyzed, only four accomplish with the phylogenetic species concept, being *B. fuscescens*, *B. kockiana* and *B. pseudofuscescens* cryptic and *B. glabra* subtly distinguishable.

7. The species of *Bryoria fuscescens* agg. are revealed as the most recent speciation event known so far in lichens.

8. *Bryoria fuscescens* s. str. contains three main genepools in Europe and North Africa, two of them widely distributed, whereas one is restricted to north of Scandinavia. The genetic traits of the last one are intermediate between *Bryoria fuscescens* and *B. pseudofuscescens*.

9. The high dispersal capacities of *Bryoria fuscescens* s. str. detected here seems influenced by the presence of shared ancestral polymorphisms.

10. In the European region, the Scandinavian Peninsula, followed by Alps and Iberian Peninsula are the areas with richest genetic diversity in *Bryoria fuscescens* s. str. The genetic diversity of the populations does not correlate with the presence or absence of apothecia.

11. *Bryoria fuscescens* s. str. seems involved in an evolutionary process influenced by genetic drift towards two phenotypic groups with high levels of incomplete lineage sorting.

12. The lichenicolous fungus *Raesaenenia huuskonenii* grow on *Bryoria fuscescens* agg. independently of the morphospecies, chemotype or genepool.

13. The *Bryoria* specimens collected in Chile belongs to an undescribed species here proposed as *Bryoria araucana*.

14. *Bryoria mariensis* should be considered a synonym of *Pseudephebe minuscula*.

15. *Pseudephebe minuscula* is a very variable species with a phenotype overlapped with *P. pubescens*, and for this, both species should be considered cryptic.

Anexos

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Short Communication

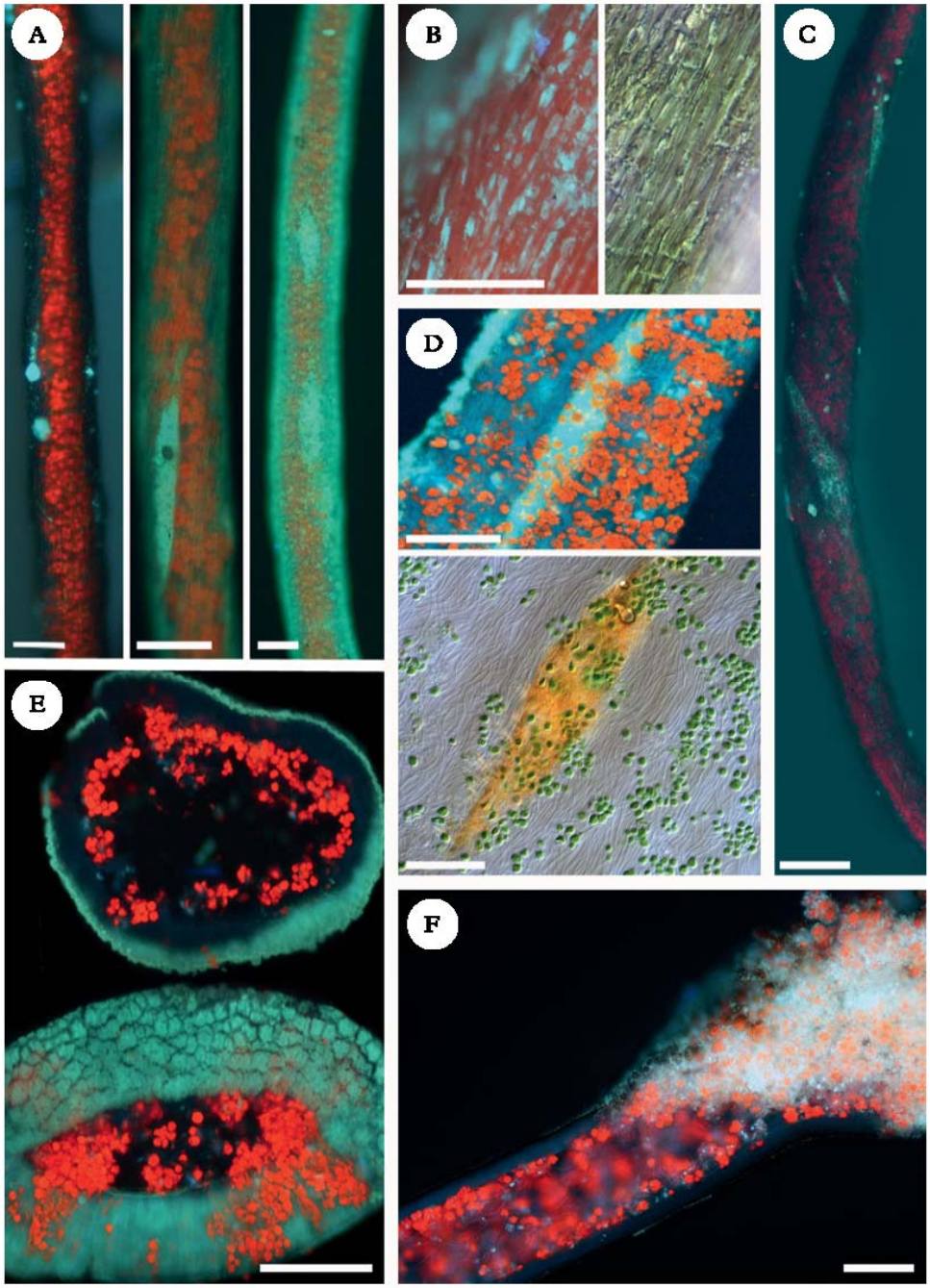
Fluorescence microscopy as a tool for the visualization of lichen substances within *Bryoria* thalli

In some species of *Bryoria* (*Parmeliaceae*), including *Bryoria bicolor* (Ehrh.) Brodo & D. Hawksw. and *B. fuscescens* (Gyeln.) Brodo & D. Hawksw., thallus reactions with spot tests are patchy, and phrases such as "Pd+ bright red at least in parts" have been used in descriptions for many years (e.g. Hawksworth 1972). It has recently been discovered that the extracellular lichen substances ('extrolites') in individual thalli of some species in the genus are more varied than hitherto assumed (Hawksworth *et al.* 2011; Myllys *et al.* 2011). We hypothesized that this variation in extrolites and the patchiness phenomenon could be due to one or more of several factors, including: 1) chemosyndromic variation; 2) variations in concentration and the sensitivity of detection methods; 3) differences between basal, median and apical regions of the thallus; or 4) the localization of particular compounds in particular anatomical features, such as pseudocyphellae and soralia. As the localization of compounds is known to occur in at least one species of the genus, *viz.* the yellow pigment vulpinic acid in the soralia of *Bryoria fremontii* (Brodo & Hawksworth 1977), we decided to explore the last possibility first.

Long-wave ultra-violet (UV) fluorescence (at 350 nm) has proved a valuable tool in the separation of thalli of similar crustose and macrolichens, and is also used routinely in the examination of thin-layer chromatographic (TLC) plates; xanthones fluoresce shades of yellow, orange and red, while depsides and depsidones generally fluoresce blue to white or shades of grey, although atranorin gives a yellow hue (Orange 2010). In addition, fluorescence microscopy has been used to explore the location of lichen products in sections of a range of macrolichens (Kauppi & Verseghy-Patay 1990). We therefore decided to explore whether fluorescence microscopy could be used to determine the localization of extrolites in whole lichen thalli, as a supplement to reagent tests, especially as material subjected to Pd reactions has to be discarded. In addition, we wished to determine whether fluorescence would disclose sites: 1) not revealed by reagent tests, that is sites where compounds were present in concentrations too low to yield visible spot test reactions; and 2) which fluoresced in different colours, suggesting the presence of more than one compound.

For this pilot investigation, we used specimens from populations of *Byoria* sect. *Implexae* (Myllys *et al.* 2011) collected in several European countries, but especially in the central mountains of Spain (deposited in MAF-Lich). We examined transverse and longitudinal sections cut by hand, and also thallus portions, mounted in water. Spot tests and TLC were performed using standard methods and solvents A, B, C, and G (Orange *et al.* 2010). For auto-fluorescence we used a Nikon microscope: D-LF epi-fluorescence module coupled to an Eclipse-80i, with bright field and DIC, and connected to a DS-Fi1 camera and DS-C2 control unit. Two filter blocks were used: Nikon UV-2A (Ex 330–380 nm, DM 400, BA 420) and B-2A (Ex 450–490 nm, DM 505, BA 520).

The most striking fluorescence was the red under the UV-2A and B-2A blocks, attributable to the chlorophyll of the included *Trebouxia* cells. This fluorescence is widely used in plant and lichen physiology as an indicator of the condition of chloroplasts and algal cells (Maxwell & Johnson 2000; Jensen 2002; Jensen & Kricke 2002). Red fluorescence, therefore, disappears in old collections (e.g. MAF-Lich. 584 from Madrid collected in 1973, MAF-Lich. 586 from


A
B
C
D
E
F

Navarra collected in 1984, MAF-Lich. 4248 from British Columbia collected in 1994). We also found that if fresh thalli were exposed to the UV sources for just 15 min, the red fluorescence becomes white, indicating chlorophyll damage. Furthermore, some regions of thallus branches were whitish blue in the algal layer instead of red, which suggests algae in some parts of thalli may be dying. Interestingly, in low-magnification bright-field microscopy, the same algae are not significantly damaged.

A bluish green fluorescence under UV-2A was evident in the granular outer layer (Fig. 1B). This consisted of small granules when sparse, but a cracked film when abundant. This variation in the surface features of *Byoria* sect. *Implexae* specimens is evident in scanning electron micrographs (SEM; Hawksworth 1969: figs 1a–d, 2a–c). The granules are extracellular and develop at a short distance behind the growing tips, and can be removed by treatment with KOH (K) and the lipase/protease enzymes in biological washing powders (Greenhalgh & Whitfield 1987). Rikkinen (1995) postulated that these granules might have a light-scattering function. *Bryoria fuscescens* can have an almost black to an almost white cortex. Under fluorescence microscopy, we found that in dark thalli this substance was hardly evident (Fig. 1A left), while in whitish thalli the granules covered the entire cortex which fluoresced bluish green (Fig. 1A right). Our material of *Bryoria capillaris* (Ach.) Brodo & D. Hawksw., with a whitish grey thallus, always fluoresced intensely bluish green in the granular outer layer (Fig. 1E). This granular fluorescent substance was more abundant in older parts of the thalli, and rarer or absent in the tips, an observation consistent with those of Greenhalgh & Whitfield (1987).

Protocetraric/fumarprotocetraric, norstictic/connorstictic, and psoromic acids gave variations of a whitish blue to greenish blue sequence of fluorescence colours, not clearly differentiated. However, the autofluorescence did serve to demonstrate extrolite distribution in the thallus using three consecutive portions of one branch of a specimen with only one TLC-detected extrolite: 1) TLC, to identify the unique lichen substance; 2) autofluorescence (UV-2A) before and after a K spot test; and 3) extrolite removal with an acetone bath (2 h at 20°C), then autofluoresence (UV-2A) before and after a K spot test. An example is specimens of *Bryoria fuscescens* with norstictic acid confined to soralia or pseudocyphellae with whitish blue fluorescence (Fig. 1D top). Adding K after fluorescence observation, the typical red acicular crystals formed in these laciniae areas, while other parts of the thallus gave no reaction (Fig. 1D bottom). After removing norstictic acid with acetone and adding K, the red crystals of the reaction were not observed by bright-field microscopy, indicating the acid had been removed. However, when that sample was examined under fluorescence, a less intense whitish blue colour remained, suggesting that either a little of the acid remained or there was another fluorescent substance present not soluble in acetone. Pseudocyphellae and soralia fluoresced the brightest (Fig. 1C & F). Further tests

FIG. 1. *Bryoria* auto-fluorescence, using UV-2A cube (except in B right and D down) and DIC. Red fluorescent colours are produced by *Trebouxia* chlorophyll. A, *Bryoria fuscescens*, granular outer layer: left, black specimen (Spain, Canary Islands, Gran Canaria, MAF-Lich. 18859); centre, brown specimen (Spain, Asturias, Caso, MAF-Lich. 18860); right, pale grey specimen (Spain, Canary Islands, Tenerife, MAF-Lich. 18861). Fusiform brightest areas correspond to an inconspicuous pseudocyphella and circular bright areas to an incipient soralium; B, *B. fuscescens*, dark olive specimen granular outer layer with (left) and without fluorescence (Spain, Madrid, MAF-Lich. 18862); C, *B. fuscescens*, dark grey lacinia with bluish fluorescent pseudocyphellae (Spain, Segovia, MAF-Lich. 18863); D, *B. fuscescens* (Spain, Madrid, MAF-Lich. 18862), dark olive specimen with inconspicuous pseudocyphellae, containing norstictic acid as unique lichen compound (TLC): up, with epi-fluorescence; down, acicular crystals after adding K, without fluorescence; E, *B. capillaris*, cross-sections of a pale grey specimen, showing the granular outer layer (Spain, Segovia, MAF-Lich. 18865); F, *B. fuscescens*, longitudinal lacinia section of a dark grey specimen with brightly fluorescent soralium (Spain, Segovia, MAF-Lich. 18864). Scales = 100 μm.

using the described combined method showed that this fluorescence was not exclusively attributable to substances detectable by TLC. This phenomenon was not restricted to *Bryoria* sect. *Implexae*. We also studied specimens of *Bryoria bicolor*, which accumulate fumarprotocetraric acid in the thallus but not in the pseudocyphellae (Pd−). Under the fluorescence microscope, the pseudocyphellae fluoresced brightly with the same colour as those of *B. fuscescens*. This suggests that this localized fluorescence in the absence of the acids is attributable to different substances. We speculated if hydrophobins could be the cause, peptide-containing proteins that self-assemble on the surfaces of hyphae, but these are not expected to fluoresce, unless labelled (Wang *et al.* 2002). The nature of this fluorescent material remains obscure and merits detailed study.

In summary, this preliminary investigation into the possibilities of the use of fluorescence microscopy for the localization of extrolites in *Bryoria* thalli, indicates that it has the potential to precisely demonstrate heterogeneous deposition sites. However, at least with the method and UV-blocks used and the compounds accumulated in *Bryoria*, the colours produced were not sufficiently distinctive to enable particular lichen products to be differentiated by eye, though it is probable that they could be separated spectrophotometrically. Furthermore, the method evidently has to be used with caution as we discovered areas of localized fluorescence where lichen acids were absent or had been eluted, notably granules on the surface, soralia, and pseudocyphellae.

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Carlos G. Boluda, Víctor J. Rico and David L. Hawksworth

C. G. Boluda, V. J. Rico and D. L. Hawksworth: Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza de Plaza Ramón y Cajal s/n, Madrid 28040, Spain. Email: carlos.g.boluda@gmail.com

D. L. Hawksworth also: Department of Life Sciences, The Natural History Museum, Cromwell Road, London SW7 5BD; and Mycology Section, Royal Botanic Gardens, Kew, Surrey TW9 3DS.

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Molecular sequence data from populations of *Bryoria fuscescens* s. lat. in the mountains of central Spain indicates a mismatch between haplotypes and chemotypes

Carlos G. BOLUDA, Víctor J. RICO, Ana CRESPO, Pradeep K. DIVAKAR and David L. HAWKSWORTH

Abstract: In order to confirm and investigate the extent of reported mismatches between chemotypes and molecular sequence data in *Bryoria fuscescens* s. lat., we examined 15 morphologically similar thalli from each of three *Pinus* forest sites in the Sistema Central of central Spain. Three thalli were rejected due to infections by *Phacopsis huuskonenii* (not previously published from Spain). The remaining 42 thalli represented nine ITS rDNA haplotypes and four chemotypes (by TLC): fumarprotocetraric and protocetraric acids; norstictic and connorstictic acids; psoromic acid; and fumarprotocetraric, protocetraric and psoromic acids. The molecular phylogenetic tree was characterized by extremely short branch lengths, often only with a single mutational difference, and a single haplotype could have different chemical products. In some cases, adjacent specimens represented different chemotypes, and three thalli appeared to be mixed individuals. Consistency of both molecular and chemical data within individual specimens was demonstrated by examining four different parts of each thallus, which showed only a difference in the location of psoromic acid in some. This is the first population-level study of this taxon, and so it is premature to propose taxonomic changes at this time. Further populations in different parts of the geographical range of this widespread complex now need to be analyzed, and more sensitive chemical analyses conducted, in order to understand the basis of the variability and determine the appropriate taxonomic treatment.

Key words: genetic diversity, haplotype network, lichens, *Parmeliaceae*, molecular phylogeny, taxonomy

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Introduction

Bryoria fuscescens s. lat., as understood in Europe, is a taxonomically difficult group composed of morphologically similar lichens which have been named as *B. chalybeiformis*, *B. fuscescens*, *B. implexa*, *B. lanestris*, and *B. subcana*. Although each of these species has been differentiated by chemical and morphological features, specimens with intermediate characters are frequent and prevent confident identifications. Preliminary molecular phylogenetic studies on material morphologically conforming to *Bryoria fuscescens* from the mountains in central Spain and Turkey, conducted in 2007, suggested a mismatch with the chemotypes as revealed by thin-layer chromatography (Hawksworth *et al.* 2011). Independently, Myllys *et al.* (2011*a*), from studies based on material from a wide geographical range rather than discrete populations, reported a similar mismatch and obtained an unresolved phylogenetic tree for some *Bryoria* sect. *Implexae* species, and subsequently suggested that many of the recognized species were conspecific (Myllys *et al.* 2011*b*).

Chemistry has traditionally been emphasized in species separations in *Bryoria*, and was used in major treatments in the 1970s (e.g. Brodo & Hawksworth 1977). The prevailing view, as noted by Krog (1980), was that

C. G. Boluda, V. J. Rico, A. Crespo, P. K. Divakar and D. L. Hawksworth: Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza de Ramón y Cajal s/n, Madrid 28040, Spain. Email: carlos.g.boluda@gmail.com
D. L. Hawksworth also: Department of Life Sciences, The Natural History Museum, Cromwell Road, London SW7 5BD, UK; and Mycology Section, Royal Botanic Gardens, Kew, Surrey TW9 3DS, UK.

"morphological plasticity in the genus *Bryoria* makes it necessary to focus on chemical characters in the final delimitation of the species". By the late 1980s, however, it was starting to become evident that some chemotypes should not be separated as different species (Holien 1989). Certain specimens, however, are now being found which contain a range of extrolites[1], such as psoromic, norstictic or fumarprotocetraric acids, which are chemically very similar. Caution in the use of structurally very similar compounds in species separation has previously been expressed (Hawksworth 1976; Lumbsch 1998). Molecular sequence data now afford a method of assessing phylogenetic relationships independently from morphological and chemical characters. As experience with other lichens in *Parmeliaceae* has demonstrated, intensive sampling of populations is necessary to fully understand their variability (e.g. Del-Prado *et al.* 2011). In order to determine whether chemistry was indeed a robust character for species delimitation in *Bryoria fuscescens* s. lat., we investigated the relationship between chemotype (the suite of extrolites in a specimen) and genetic kinship as inferred by the ITS rDNA sequences in three populations conforming morphologically to *Bryoria fuscescens* s. str., from the mountains of the Sistema Central in Spain. Furthermore, in order to ascertain if there was variation in the chemical products detected in different parts of the specimens, or if single specimens were of intermixed genotypes or "mechanical hybrids" (Hawksworth 1988), we separated each specimen into four different portions which were examined separately.

Materials and Methods

Three populations conforming morphologically to *Bryoria fuscescens* s. str., collected in the Sistema Central mountains of Spain, were studied. All specimens sampled had dark coloured thalli, paler basal parts, acute branching angles, pseudocyphellae which were inconspicuous or absent, and fissural as well as tuberculate soralia. In collecting, care was taken to avoid other *Bryoria* species or specimens with different morphological characteristics:

1) *Segovia*: La Granja de San Ildefonso, Sierra de Guadarrama, between Puerto de Cotos and Puerto de Navacerrada, 40°47'34·97"N / 03°59'12·62"W, 1854 m, 25 May 2012, *C. G. Boluda & V. J. Rico* (MAF-Lich. 18863, 18865, 18923-18932; GenBank accession numbers KJ652402 to KJ652413).
2) *Madrid*: Navacerrada, Sierra de Guadarrama, La Barranca, 40°46'06·3"N / 03°59'04"W, 1580 m, 11 July 2012, *C. G. Boluda & V. J. Rico* (MAF-Lich. 18862, 18933-18946; GenBank accession numbers KJ652414 to KJ652428).
3) *Avila*: Navarredonda de Gredos, Sierra de Gredos, Pinar de Navarredonda, near the Parador Nacional de Gredos, 40°21'10"N / 05°06'45"W, 1550 m, 1 July 2012, *V. J. Rico* (MAF-Lich. 18947 to 18961; GenBank accession numbers KJ652429 to KJ652443).

All three sites were of uneven-aged *Pinus sylvestris* forests over granite, and the specimens were restricted to mature *P. sylvestris* trunks. The lichen community belonged to the *Pseudevernion furfuraceae* (James *et al.* 1977), and was dominated in these sites by *Hypogymnia farinacea*, *Parmelia serrana*, *P. sulcata*, *Platismatia glauca*, *Pseudevernia furfuracea*, and less abundantly *Tuckermannopsis chlorophylla*.

Fifteen discrete specimens were collected in each site. Three of those from the Madrid locality, however, were subsequently rejected due to the presence of the lichenicolous fungus *Phacopsis huuskonenii*, a species not previously published as occurring in Spain. For each of the remaining 42 samples, four thallus regions were cut and examined separately: 1) the base (the oldest part, usually in contact with the bark); 2) the median zone (middle of the branches, but with soralia removed); 3) the tips (the last 5 mm of the branches); and 4) the soralia. In total, 168 subsamples (42 × 4) were analyzed, using the same material for TLC and DNA extraction.

For the phylogenetic tree reconstruction, *Bryoria glabra* (Finland, GenBank accession number HQ402725.1) was used as outgroup (Myllys *et al.* 2011*a*).

Extrolite chemistry

Spot tests were made using C, K, KC, and Pd, and TLC was performed using standard methods (Orange *et al.* 2010). For the TLC, concentrated lichen extractions in acetone were spotted onto silica gel 60 F_{254} aluminium sheets (Merck, Darmstadt) and run with the solvents A, B, C and G. Spots were visualized under UV and after a sulphuric acid spray.

Molecular and bioinformatics techniques

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Barcelona) with a slight modification to the

[1]"Extrolite" refers to all compounds which are secreted from fungal hyphae, and was first used by Frisvad (2005). "Secondary metabolites" is an inappropriate term as these are not metabolized but are often products with ecological roles.

manufacturer's instructions (Crespo *et al.* 2001). The fungal ITS rDNA region was amplified using the primers ITS1FKYO2 (TAG AGG AAG TAA AAG TCG TAA) and ITS4KYO2 (RBT TTC TTT TCC TCC GCT) (Toju *et al.* 2012). For amplification, we used a reaction mixture of 25 µl, containing 18 µl of sterile water, 2·5 µl of 10 × buffer with 2 mM $MgCl_2$, 0·5 µl dNTPs (10 mM of each base), 1·25 µl of each primer at 10 µM, 0·625 µl of DNA polymerase (1U μl^{-1}), and 5 µl of diluted 1/10 DNA template. For any failed samples the PCR was repeated using PuReTaq Ready-To-Go PCR Beads (2·5 U of PuReTaq DNA Polymerase, 200 µM of each dNTP, BSA, buffer reaction and stabilizers: 10 mM Tris-HCl pH 9·0, 50 mM KCl, 1·5 mM $MgCl_2$; GE Healthcare, Little Chalfont, UK), adding to the lyophilized bead 20 µl of sterile water, 1 µl of each primer at 10 µM and 3 µl of diluted 1/10 DNA template.

The amplifications were run in an automatic thermocycler (XP Cycler, Bioer, Hangzhou) using the following parameters: initial denaturation 5 min at 95 °C, then 35 cycles of 1 min at 95 °C, 1 min at 56 °C, 1·5 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products were cleaned using illustra™ ExoProStar (GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. Sequencing was performed by the Unidad de Genómica (Parque Científico de Madrid) and Stabvida (Lisbon, Portugal).

DNA sequences obtained were manually adjusted using SeqMan version 7.0 (DNAstar, Madison) and MEGA5 (Tamura *et al.* 2011). For genetic analyses, only one sequence per specimen instead of four was used, selecting the sequences belonging to the basal portion as those would be of the haplotype when the thallus started to grow. The alignment was performed using MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/; Katoh & Standley 2013), using G-INS-I alignment algorithm, a scoring matrix of 1PAM/k = 2, and offset value of 0·1. Gblocks version 0.91b (Barcelona; http://molevol.cmima.csic.es/castresana/Gblocks_server.html) was used to delete non-conserved GAPs, allowing smaller final blocks, gap positions within the final blocks, and less strict flanking positions, which resulted in the elimination of a single gap in the outgroup. The alignment was analyzed using maximum likelihood (ML) and Bayesian (B/MCMC) approaches. For the maximum likelihood (ML) tree reconstruction, we used the program RAxML v7.2.8 (Stamatakis 2006). The GTRGAMMA model was applied, which includes a parameter (Γ) for rate heterogeneity among sites, and we chose not to include a parameter for estimating the proportion of non-variable sites (Stamatakis 2006; Stamatakis *et al.* 2008). Analysis was performed using RAxML v7.2.8, as implemented on the CIPRES Science Gateway (http://qball2.sdsc.edu:7070/portal2/home.action; Miller *et al.* 2010) with the GTRGAMMA model as described above. Support values were assessed using the 'rapid bootstrapping' option with 1000 replicates. For the Bayesian reconstruction, MrBayes v3.2.1 (Ronquist & Huelsenbeck 2003) was used. The analysis was performed assuming the general time reversible model (Rodríguez *et al.* 1990), assuming a discrete gamma distribution with six rate categories (GTR + G). The nucleotide-substitution model and parameters were selected using the Akaike Information Criterion as implemented in jModelTest (Posada 2008). A run with four million generations, starting with a random tree and employing eight simultaneous chains, was executed. Every 400th tree was saved to a file. We plotted the log-likelihood scores of sample points against generations using TRACER v1.5 (Rambaut & Drummond 2007) and determined that stationarity had been achieved when the log-likelihood values of the sample points reached an equilibrium value (Huelsenbeck & Ronquist 2001), discarding the trees obtained before stationarity was reached. Posterior probabilities (PPs) were obtained from the 50% majority-rule consensus of sampled trees after excluding the initial 25% as burn-in. The phylogenetic tree was drawn using FigTree v1.4 (Rambaut 2009).

For the haplotype network reconstruction, TCS v1.2.1 was employed, using gaps as missing data and 95% as the connection limit. We used DnaSP v4.50 (Librado & Rozas 2009) to calculate estimates of genetic diversity. PAUP 4.0 was used to calculate the haplotype diversity, number of polymorphic sites, nucleotide diversity, Tajima's D value, Fu's F statistic, and the raggedness index. For genetic comparison among predefined groups, the AMOVA test was implemented in Arlequin v3.5 (Excoffier *et al.* 2005), comparing differences among chemotypes and among populations. In order to test if there was a definite correlation between chemotypes and haplotypes, we performed a Fisher's exact test implemented using the R package (R Development Core Team 2012).

Results

Chemical investigations

Four extrolite profiles were found: 1) norstictic and connorstictic acids; 2) fumarprotocetraric, protocetraric, and psoromic acids; 3) protocetraric and fumarprotocetraric acids only; and 4) psoromic acid only (Table 1). We did not detect atranorin in this study; this compound is known to occur sporadically in various *Bryoria* species (e.g. Myllys *et al.* 2011*b*) but is generally at low concentrations and not found in routine TLC. Although the three populations were in the same macroenvironment, there were marked differences in the percentage abundance of each chemotype. In some cases, two specimens collected close together, and apparently in the same microenvironment, had different extrolite profiles.

TLC did not reveal differences in the presence /absence of extrolites in the four thallus regions, nor between the soralia and other parts of the thallus, except in two specimens. One had protocetraric, fumarprotocetraric, and psoromic acids in all parts of the thallus,

TABLE 1. *Chemotype frequencies in the 42 specimens of* Bryoria fuscescens *examined from 3 separate populations collected in 3 localities.*

	Chemotype frequencies			
Locality	N	F	P	FP
Segovia	7	2	6	0
Madrid	4	8	0	0
Ávila	2	1	10	2

N = norstictic and connorstictic acids, F = fumarprotocetraric and protocetraric acids, P = psoromic acid, FP = fumarprotocetraric, protocetraric and psoromic acids

except that the base lacked psoromic acid, while the other contained protocetraric and fumarprotocetraric acids except for the soralia which additionally contained psoromic acid. Although TLC indicates a homogeneous extrolite distribution along the thallus (with the exception of these two specimens), this may not be conclusive, as autofluorescence studies indicate that there can be chemical heterogeneity within thallus portions (Boluda *et al.* 2014). For example, norstictic acid is commonly only present in soralia and inconspicuous pseudocyphellae, while fumarprotocetraric acid can be restricted to soralia. TLC of acetone extracts from thallus portions cannot detect such small-scale heterogeneic distributions. The results in the current study therefore have to be interpreted as indicating that, while there is generally no variation in extrolite composition from the base to the tips, they cannot exclude the possibility of heterogeneous small-scale distribution within thallus portions.

Molecular investigations

The amplified PCR products obtained were around 800 bp. Usually this PCR product is about 600 bp; the difference in size found in our samples was due to the presence of insertions of about 200 bp identified as group I introns (Gutiérrez *et al.* 2007) at the 3' end of the SSU rDNA. We excluded group I introns as well as the SSU and LSU neighbouring regions of the ITS from the analysis.

The ITS sequences of the four thallus portions of each of the 42 specimens (42 × 4) revealed three cases of intra-thalline diversity (7·14% of the samples). These specimens were composed of two different genotypes, which were also present in other specimens from the same populations. In one specimen, the genotypes alternated among the four thallus portions, but in the other two the median zone contained a different genotype. In these three specimens, the extrolites were the same in all four segments tested. This result suggests that what seems to be a discrete and independent thallus can be composed of two or more intermixed genotypes.

The sequences used for tree and haplotype network reconstruction (one per specimen) contained a haplotypic diversity (Hd) of 0·850, seven polymorphic sites (S), a nucleotide diversity (π) of 0·00331, a Tajima's D value of −0·4847 ($P > 0·10$, not significant), a Fu's F statistic of −2·519 ($P > 0·10$, ns), and a raggedness index (R) of 0·0944 ($P > 0·10$, ns) with a unimodal mismatch distribution. Each of the 42 specimens was found to belong to one of nine haplotypes. The tree reconstruction (Fig. 1) was characterized by exceptionally short branch lengths (note that the scale in Fig. 1 = 0·003 substitutions per site) compared with those seen within other species of the genus (cf. Myllys *et al.* 2011*a*). This represents a particularly low level of genetic diversity in the ITS sequences, many specimens having identical sequences. The same haplotypes occurred in the different populations, as evident from the haplotype network obtained (Fig. 2); all haplotypes were connected by a single mutation. Three of the nine haplotypes were represented by single specimens. Haplotypes represented by more than one specimen (except haplotype 2 with two specimens), also included more than one chemotype. Three of the predominant haplotypes (numbers 5, 9 and 6, with nine, nine and five specimens, respectively) contained specimens showing in total the full range of extrolites found in this study within each haplotype (norstictic, psoromic, and fumarprotocetraric acids). AMOVA results showed that 24·7% of the genetic variation was among the four chemotypes, while the variation among the collected populations was 7·8%.

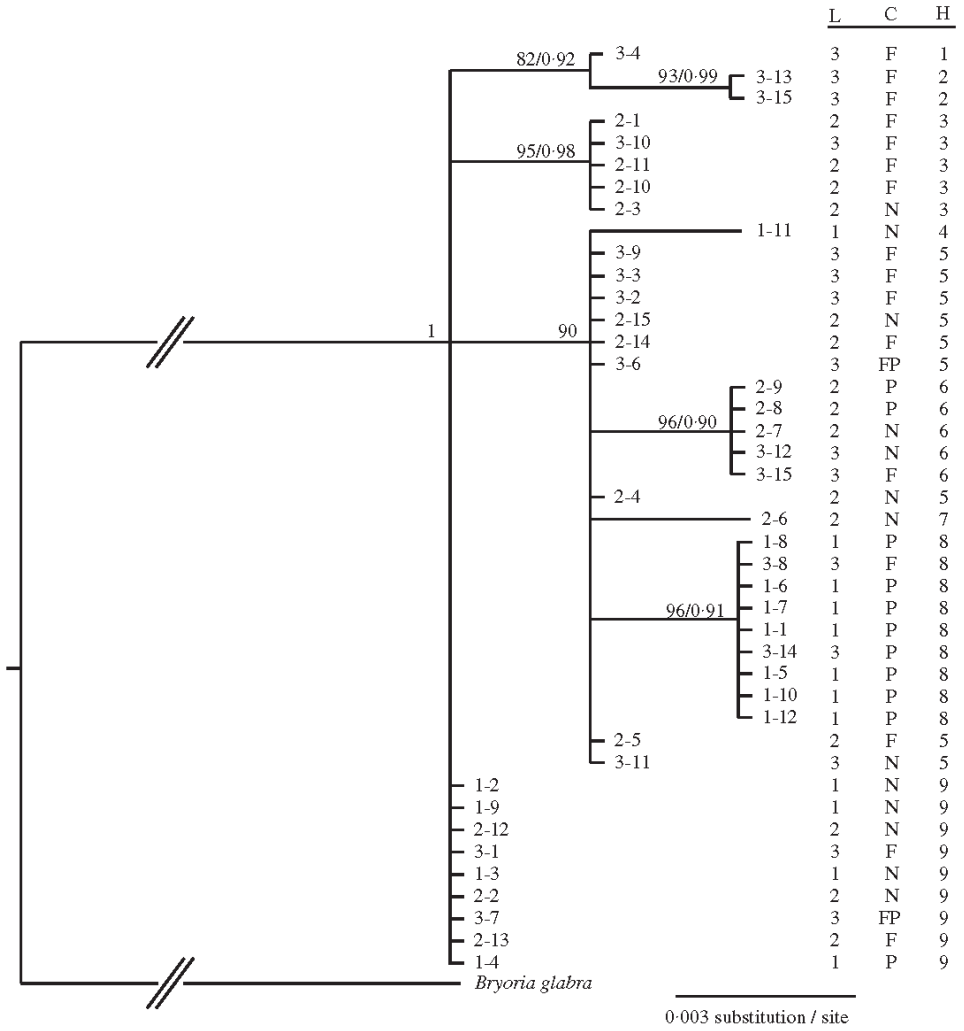

FIG. 1. Phylogram obtained from maximum likelihood analysis of ITS rDNA sequences from 3 populations of *Bryoria fuscescens* obtained from the Sistema Central Mountains of Spain. ML bootstrap values ≥75 and posterior probabilities ≥0·90 for the Bayesian analyses are indicated above the branches. The tree tip numbers indicate the locality and specimen number respectively. L = locality (1 = Segovia, 2 = Madrid, and 3 = Ávila); C = chemotype (F = fumarprotocetraric and protocetraric acids, N = norstictic and connorstictic acids, P = psoromic acid, FP = fumarprotocetraric, protocetraric and psoromic acids); H = haplotype.

The Fisher's exact test indicated that the hypotheses of there being no correlation between haplotypes and chemotypes ($P < 0{\cdot}001$), nor between haplotypes and populations ($P < 0{\cdot}005$), could not be entirely ruled out from the data available. The reason for this was that some haplotypes were represented by only one or two specimens in only one chemotype or population. In the case of the haplotypes in our study

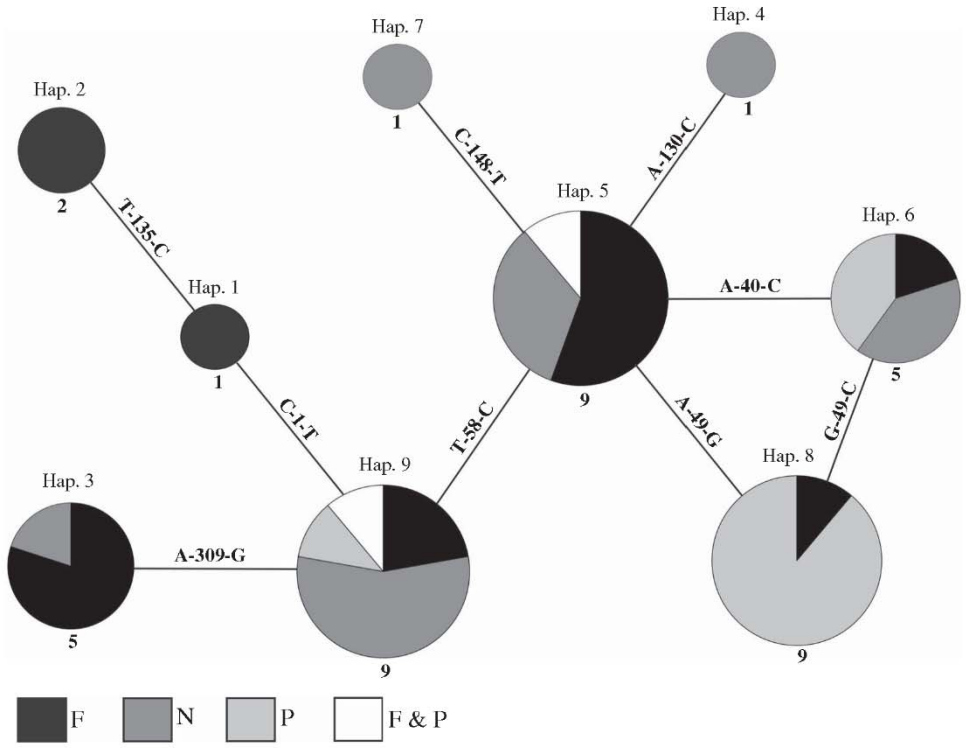

FIG. 2. 95% probability haplotype network based on ITS rDNA sequences of the 42 specimens from 3 populations of *Bryoria fuscescens* examined. The circle size is proportional to the number of specimens sharing a haplotype. Numbers below the circles indicate the number of specimens. Text on the connecting lines indicates the nucleotide substitution. Hap. = haplotype, F = fumarprotocetraric and protocetraric acids, N = norstictic and connorstictic acids, P = psoromic acid.

represented by greater numbers of specimens, the test showed that haplotypes and chemotypes were not correlated. This suggests that there is no correlation between extrolite chemistry and genetic kinship, but more samples are needed to be confident that this is the case for all haplotypes and chemotypes represented in our samples.

Discussion

Bryoria fuscescens s. lat. is taxonomically difficult to resolve and the assignment of specimens to currently recognized species can be frustrating. Our results, the first to be based on intensive molecular and chemical analyses of discrete morphologically more-or-less uniform populations conforming to *B. fuscescens* s. str. but including some deviating chemically, suggest that extrolite production, which has been emphasized in species circumscription in these lichens since the 1970s, is not correlated with particular haplotypes (phylogenetic lineages) as revealed by ITS. We also show that populations lacking apothecia and so expected to reproduce clonally, and which also appear morphologically homogeneous, can comprise a mixture of haplotypes and can vary in the TLC-detectable extrolites. We found that specimens morphologically assigned to *B. fuscescens* may not contain fumarprotocetraric acid only, as historically assumed, but are

much more variable in their chemistry. As pointed out previously (Boluda *et al.* 2014), however, a fuller picture of the extrolite patterns in the complex will require the use of high performance liquid chromatography (HPLC) to detect compounds present at lower concentrations than can be visualized by TLC from extracts of single small thallus portions, and further fluorescence microscopy to explore the localization of compounds in thalli more precisely. In addition, negative but non-significant Tajima's D value and Fu's F statistics may indicate population stability (i.e. no evidence of demographic expansion or contraction) of *B. fuscescens* in the regions studied. The unimodal curve of mismatch frequency, however, suggests population expansion or spatial range expansion, but with a non-significant raggedness index. These contradictory results may well be attributable to bias arising from the limited sample size (Ramos-Onsins & Rozas 2002), and so more comprehensive studies would be required to test that hypothesis.

We conclude that extrolite composition appears to be of limited value for the possible separation of species within material conforming morphologically, but not always chemically, to *Bryoria fuscescens*. Furthermore, as the three populations we investigated were growing in similar ecological situations and on the same species of tree, there were no evident ecological factors to which the differences observed could be attributed, nor were there associations with particular genetic lineages as revealed by the ITS rDNA region.

Similar population studies across the range of the species complex are required to determine the extent to which those of the Spanish Sistema Central are representative of the situation throughout its geographical range. Furthermore, *B. fuscescens* s. lat. is much more abundant in northern Europe, where apothecia can sometimes be found, albeit at a low frequency, while in southern Europe specimens are almost exclusively asexual and occur as isolated populations. It would therefore be of interest to ascertain if a greater range of genetic diversity occurs in more northern regions.

Without more detailed population studies over a larger geographical area, we consider it unwise to conclude that material currently named as *Bryoria capillaris*, *B. chalybeiformis*, *B. fuscescens*, *B. implexa*, *B. lanestris*, or *B. subcana* should be treated as conspecific on the basis of DNA data alone, as suggested by Myllys *et al.* (2011*b*). In order to elucidate the situation in *Bryoria fuscescens* s. lat., and lead to a more robust taxonomy for these lichens, sequences from more DNA regions and microsatellite population studies are required to determine if there are other correlations between chemistry, morphology, geography, and genetic data.

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PRIMER NOTE

CHARACTERIZATION OF MICROSATELLITE LOCI IN LICHEN-FORMING FUNGI OF *BRYORIA* SECTION *IMPLEXAE* (PARMELIACEAE)[1]

OLGA NADYEINA[2,3,6], CAROLINA CORNEJO[2], CARLOS G. BOLUDA[2,4], LEENA MYLLYS[5], VÍCTOR J. RICO[4], ANA CRESPO[4], AND CHRISTOPH SCHEIDEGGER[2]

[2]Swiss Federal Research Institute WSL, Zürcherstrasse 111, CH-8903 Birmensdorf, Switzerland; [3]M. H. Kholodny Institute of Botany, Tereschenkivska 2, Kyiv (Kiev) 01601, Ukraine; [4]Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza de Ramón y Cajal s/n, Madrid 28040, Spain; and [5]Botanical Museum, Finnish Museum of Natural History, FI-00014 University of Helsinki, Helsinki, Finland

- *Premise of the study:* The locally rare, haploid, lichen-forming fungi *Bryoria capillaris*, *B. fuscescens*, and *B. implexa* are associated with boreal forests and belong to *Bryoria* sect. *Implexae*. Recent phylogenetic studies consider them to be conspecific. Microsatellite loci were developed to study population structure in *Bryoria* sect. *Implexae* and its response to ecosystem disturbances.
- *Methods and Results:* We developed 18 polymorphic microsatellite markers using 454 pyrosequencing data assessed in 82 individuals. The number of alleles per locus ranged from two to 13 with an average of 4.6. Nei's unbiased gene diversity, averaged over loci, ranged from 0.38 to 0.52. The markers amplified with all three species, except for markers Bi05, Bi15, and Bi18.
- *Conclusions:* The new markers will allow the study of population subdivision, levels of gene introgression, and levels of clonal spread of *Bryoria* sect. *Implexae*. They will also facilitate an understanding of the effects of forest disturbance on genetic diversity of these lichen species.

Key words: Ascomycetes; *Bryoria implexa*; lichen-forming fungi; microsatellites; *Treboxia* spp.

The members of *Bryoria* sect. *Implexae* are pendent, copiously branched lichens with circumboreal distribution (Brodo and Hawksworth, 1977; Myllys et al., 2011a). They are an important component of the boreal forests (Glavich et al., 2005), and their frequency depends on forest fragmentation (Hilmo and Holien, 2002). These lichen-forming fungi are haploid and disperse with vegetative propagules; sexual reproduction with ascospores is uncommon (Brodo and Hawksworth, 1977). *Bryoria* sect. *Implexae* includes seven morphologically and chemically recognized species in Europe (Myllys et al., 2011a), which have different frequency across longitudinal and altitudinal gradients (Hawksworth, 1972; Myllys et al., 2011a). Molecular data confirm the monophyly of the section, although the relationships among the currently recognized species remain poorly understood because phylogenetic analyses suggest that several species are conspecific (Myllys et al., 2011b). Highly variable microsatellite markers of the fungal partner of lichen symbioses (Widmer et al., 2010; Devkota et al., 2014) will be used to study the genetic diversity and differentiation in *Bryoria* sect. *Implexae*, to determine the gene flow across and within the currently recognized species, and to assess the impact of land use and habitat fragmentation on population structure of these locally rare and threatened, boreal forest–associated lichens.

METHODS AND RESULTS

Eighty-two specimens representing the three morphologically and chemically characterized species, *Bryoria capillaris* (Ach.) Brodo & D. Hawksw., *B. fuscescens* (Gyeln.) Brodo & D. Hawksw., and *B. implexa* (Hoffm.) Brodo & D. Hawksw., were collected in three regions (Spain, Switzerland, and Finland; Appendix 1). All specimens are deposited in the Lichens Herbarium of the Universidad Complutense de Madrid (MAF-Lich), and duplicates are stored at the Swiss Federal Research Institute WSL at –20°C. A subset of 30 specimens was used for total DNA extraction with the MoBio PowerPlant Pro DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA). The pooled DNA was used to create a shotgun multiplex identifier library using the GS FLX Titanium Rapid Library Preparation Kit (Roche Diagnostics, Basel, Switzerland), and Microsynth AG (Balgach, Switzerland) provided the barcode adapters. The library was sequenced on 1/4th of a plate on a Roche 454 Genome Sequencer FLX at Microsynth. We obtained 533,962 reads of an average length of 812 bp (National Center for Biotechnology Information [NCBI] Sequence Read Archive [SRA] accession no. SRR1283191; http://www.ncbi.nlm.nih.gov/sra). The unassembled sequences were screened for di-, tri-, tetra-, and pentanucleotide microsatellites using MSATCOMMANDER 1.0.2 alpha (Rozen and Skaletsky, 1999; Faircloth, 2008), ensuring a minimum repeat length of 8 bp for dinucleotides and 6 bp for all others.

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[6]Author for correspondence: olga.nadyeina@wsl.ch

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MSATCOMMANDER recovered 6329 primer pairs that fulfilled the default primer parameters among all reads. Of those, 5932 pairs were discarded from further studies because they contained unfavorable secondary structure, primer-dimer formation, monorepeats in the flanking region, or because they were duplicates, which we detected after alignment using CLC Main Workbench 6 (CLC bio, Aarhus, Denmark). Putative sequences of algae, plants, animals, or microorganisms, which are often present in epiphytic samples, were identified and removed using the ntBLAST search on http://www.ncbi.nlm.gov. This inspection resulted in 58 primer pairs used for further analysis, i.e., to test for amplification with the symbiotic partner of these lichen-forming fungi. We used DNA from five axenic cultures of *Trebouxia* spp., which are hypothesized to be the photobionts of *Bryoria* sect. *Implexae* (Lindgren et al., 2014): *T. angustilobata* Beck (SAG2204), *T. asymmetrica* Friedl & Gärtner (SAG48.88), *T. arboricola* Puymaly (SAG219-1a), *T. jamesii* (Hildreth & Ahmadjian) Gärtner (SAG2103), and *T. simplex* Tschermak-Woess (SAG101.80). Forward primers were labeled with an M13 tag (5′-TGTAAAACGACGGCCAGT-3′) for PCR amplification (Schuelke, 2000). All PCR runs were performed on Veriti Thermal Cyclers (Life Technologies, Carlsbad, California, USA). The PCR reactions were evaluated in a temperature gradient with one-degree steps from 56–61°C, performed with the JumpStart REDTaq ReadyMix (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's protocol, with the following conditions: denaturation for 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 56–61°C, and 45 s at 72°C; then for the M13-tag binding additional eight cycles of 30 s at 94°C, 45 s at 53°C, and 45 s at 72°C, with a final extension of 30 min at 72°C. In total, 14 primer pairs produced positive PCR reactions with at least one of the five *Trebouxia* species, and were excluded from further analyses because they were considered alga-specific.

The amplification of the fungal component of *Bryoria* sect. *Implexae* was tested with the 44 remaining loci under the same conditions as mentioned above. There were 14 loci that produced specific single products at an annealing temperature of 56°C, 12 at 57°C, six at 58°C, six at 60°C, and six at 61°C. Polymorphism of the 44 microsatellite loci was initially tested on a subset of 12 individuals (four individuals from each of three countries: Spain, Switzerland, and Finland), resulting in 18 polymorphic loci with satisfactory amplification. All PCR products obtained were multiplexed (Table 1). PCR reactions were performed in a total volume of 10 µL containing 1 µL of ~5 ng genomic DNA, 1 µL each of forward and reverse primers of varying concentration (Table 1), and 5 µL of Type-it Multiplex PCR Master Mix (QIAGEN, Hilden, Germany). The PCR protocol used fluorescent forward primers and the reaction was adjusted to: 5 min at 95°C; followed by 30 cycles of 30 s at 95°C, 90 s at 56, 58, or 60°C (Table 1), and 30 s at 72°C; with a final extension of 60 min at 60°C. PCR products were run on a 3130xl DNA Analyzer with GeneScan 500 LIZ as the size standard for fragment analysis (both by Life Technologies).

The 18 polymorphic microsatellite markers were tested for locus variability and marker consistency on three populations (Table 2). Alleles were sized using GeneMapper 5.0 (Life Technologies). The linkage disequilibrium (LD) between microsatellite loci and their variability were measured by counting the number of alleles and calculating Nei's unbiased gene diversity using Arlequin 3.11 (Excoffier et al., 2005). Dinucleotide microsatellites ($n = 13$) were the most common microsatellite motifs among the 18 loci (Table 1). The microsatellite loci revealed significant LD based on 999 permutations ($P < 0.001$). They show two to 13 alleles per locus with a mean of 4.6, and average gene diversities varied from 0.38 to 0.52 over three populations (Table 2).

TABLE 1. Overview of the microsatellite loci developed for the group of lichen-forming fungi *Bryoria* sect. *Implexae*.

Locus	Primer sequences (5′–3′)	Repeat motif	Multiplex[a]	T_a (°C)	Fluorescent dye	Primer conc. (µM)	Allele size range (bp)	GenBank accession no.
Bi01	F: GGACGACGACATACCACTC R: GAGTTCGGGTTTAGGTCGTC	$(AACAGC)_6$	1	56	FAM	0.32	94–129	KJ739845
Bi02	F: GCGTGAATGTGTCCGAATCG R: GAATGGGCGCTCACTGTCTT	$(AG)_{12}$	1	56	FAM	0.80	369–372	KJ739846
Bi03	F: GTGAACTCGCTCGTATCGTC R: CCTAGGGATGACACGCAGAA	$(AG)_{12}$	1	56	FAM	0.80	279–281	KJ739847
Bi04	F: CAGTGCGGCAAACAGTTAGT R: GCACAAATCCACCCACTCCT	$(TG)_{10}$	1	56	PET	0.80	320–325	KJ739848
Bi05	F: CAAGGAGGTCGACTGTGAGT R: CAACCGATCCCACGCTCTC	$(AAGG)_6$	1	56	NED	0.50	127–143	KJ739849
Bi06	F: GGGAGGGTGGAAGTTGGTTT R: CGACCACTTCCACTTCCATATC	$(GTT)_9$	1	56	PET	0.32	114–168	KJ739850
Bi07	F: GAAATCGGCTTGTTGTCCTCC R: GAACTACCGCCCACAAACAA	$(CCTTT)_6$	2	58	PET	0.80	123–144	KJ739851
Bi08	F: CATGCGGAGTTAAAGGAGGC R: CGCACCTATTTACGGCCTTT	$(TC)_8$	2	58	NED	0.32	367–372	KJ739852
Bi09	F: CGTTCGTTCGTAGGTAGGTA R: GCCTACCCACCATCTGAACT	$(AT)_8$	2	58	PET	1.10	341–343	KJ739853
Bi10	F: CTCGCGTTTCCCTGTTTCTT R: GTATGAGGTCGGAGTGTGCT	$(TC)_8$	2	58	FAM	0.90	434–437	KJ739854
Bi11	F: GCACAAATCCACCCACTCCT R: CAGTGCGGCAAACAGTTAGT	$(AC)_{12}$	2	58	FAM	0.50	314–318	KJ739855
Bi12	F: GCAGAAAGTGAGTTAGCCGG R: CTCAGCCTCAACCACAACGA	$(TTG)_{12}$	2	58	FAM	0.32	100–124	KJ739856
Bi13	F: TCTTTCCTCTCCTGTCCACC R: CCTTACAGACCGGAGAAGCC	$(TTC)_{11}$	3	60	FAM	0.90	93–134	KJ739857
Bi14	F: CTAACCACGACAAGCTGACC R: GTACCGACGCAACTTACCTA	$(TC)_7$	3	60	FAM	0.60	316–365	KJ739858
Bi15	F: GTAGCAGGACATACGGAGGT R: CGTCCTAGCATCTCGGTTCT	$(TC)_9$	3	60	PET	3.00	379–381	KJ739859
Bi16	F: CCAGGTCCTTCACTACAGCT R: CGGTACAAGTCCAGTTGCAG	$(AG)_8$	3	60	FAM	1.50	405–437	KJ739860
Bi18	F: GCAGCTATCAGGAGTCACGT R: GCAGCTATCAGGAGTCACGT	$(TC)_7$	3	60	VIC	0.60	387–396	KJ739861
Bi19	F: CCACCTCGAAGAGTACTGCT R: CTGAGCTATGTCCTCGCACA	$(TC)_{10}$	3	60	PET	0.80	346–352	KJ739862

Note: T_a = annealing temperature.
[a]Multiplex indicates loci that were mixed in the same capillary electrophoresis run.

TABLE 2. Results of microsatellite screening in 82 individuals of lichen-forming fungi of *Bryoria* sect. *Implexae* between species of *Bryoria* sect. *Implexae*, and between compared regions.

	Total			*B. capillaris* ($n = 36$)		*B. fuscescens* ($n = 37$)		*B. implexa* ($n = 9$)		Spain ($n = 31$)		Switzerland ($n = 35$)		Finland ($n = 16$)	
Locus	n	A	H_e	A	H_e	A	H_e	A	H_e	A	H_e	A	H_e	A	H_e
Bi01	82	7	0.82	6	0.71	6	0.79	4	0.58	5	0.73	6	0.71	4	0.44
Bi02	67	4	0.74	3	0.64	4	0.68	2	0.43	3	0.68	4	0.69	3	0.59
Bi03	82	2	0.24	2	0.32	2	0.15	2	0.22	2	0.12	2	0.36	2	0.13
Bi04	82	3	0.36	3	0.45	2	0.28	2	0.39	2	0.12	3	0.54	2	0.33
Bi05	79	4	0.61	3	0.57	3	0.47	2	0.22	3	0.52	4	0.66	2	0.13
Bi06	82	10	0.83	10	0.88	5	0.64	3	0.64	3	0.53	8	0.85	4	0.64
Bi07	82	3	0.49	3	0.37	2	0.11	1	0.00	2	0.28	3	0.46	2	0.13
Bi08	82	4	0.54	3	0.52	3	0.49	3	0.56	2	0.49	3	0.54	3	0.57
Bi09	60	2	0.50	2	0.25	2	0.28	1	0.00	2	0.40	2	0.31	2	0.33
Bi10	82	2	0.44	2	0.44	2	0.05	1	0.00	2	0.23	2	0.49	2	0.13
Bi11	82	3	0.36	3	0.45	2	0.28	2	0.39	2	0.12	3	0.54	2	0.33
Bi12	82	7	0.67	5	0.39	6	0.49	4	0.81	3	0.34	6	0.48	5	0.82
Bi13	82	13	0.84	9	0.80	8	0.68	6	0.92	6	0.67	9	0.83	7	0.88
Bi14	82	3	0.47	3	0.40	2	0.05	1	0.00	2	0.23	3	0.48	2	0.13
Bi15	52	2	0.04	1	0.00	2	0.05	1	0.00	1	0.00	2	0.13	1	0.00
Bi16	82	6	0.76	6	0.57	5	0.61	3	0.72	3	0.61	6	0.67	4	0.69
Bi18	81	4	0.56	4	0.35	3	0.62	3	0.68	3	0.59	4	0.27	3	0.68
Bi19	82	3	0.65	3	0.11	3	0.53	3	0.72	3	0.60	3	0.43	3	0.69
Mean		4.58	0.53	6	0.71	6	0.79	4	0.58	2.63	0.38	4.11	0.52	2.84	0.40

Note: A = number of alleles; H_e = Nei's unbiased gene diversity; n = total number of samples analyzed.

Cross-species amplifications within three congeneric species were analyzed with the chi-square test; *B. capillaris* was shown to not amplify consistently, while *B. fuscescens* and *B. implexa* amplified more regularly (Appendix 2). Most markers amplified with all three species. However, the microsatellite marker Bi15 only amplified with *B. fuscescens*, Bi05 with *B. fuscescens* and *B. implexa*, and Bi18 with *B. capillaris* and *B. fuscescens*.

CONCLUSIONS

The fungus-specific markers developed here will facilitate studies on genetic diversity and differentiation in *Bryoria* sect. *Implexae* throughout its geographic distribution, and on effects of forest management on genetic diversity of populations in this species group. Furthermore, putative phylogenetic signal within the flanking regions of the microsatellite sequences might help to delimit closely related species and to assess the taxonomic value of the morphological and chemical characters of these regionally rare and threatened lichens.

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APPENDIX 1. Voucher information for species of *Bryoria* sect. *Implexae* used in this study.

Species	Voucher specimen accession no.[a]	Collection locality and date	Geographic coordinates	No. of individuals
B. capillaris	18964–18967	Spain, Prov. Segovia, 1854 m a.s.l., *Pinus sylvestris* forest, 6 Nov. 2012	40°47′35.0″N, 03°59′12.6″W	4
B. capillaris	18968–18993	Switzerland, Canton of Berne, 1511 m a.s.l., *Picea abies* forest, 25 Nov. 2012	46°35′28.3″N, 07°20′26.9″E	26
B. capillaris	18997–18999	Finland, Prov. Etelä-Häme, Liesjärvi, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012	60°40′17.0″N, 23°51′10.4″E	3
B. capillaris	18994–18996	Finland, Prov. Etelä-Häme, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012	60°42′04.3″N, 23°54′41.9″E	3
B. fuscescens	19001–19014	Spain, Prov. Madrid, 1490 m a.s.l., *Pinus sylvestris* forest, 6 Nov. 2012	40°46′05.4″N, 03°59′35.9″W	14
B. fuscescens	19015–19027	Spain, Prov. Segovia, 1854 m a.s.l., *Pinus sylvestris* forest, 6 Nov. 2012	40°47′35.0″N, 03°59′12.6″W	13
B. fuscescens	19028–19034, 19036	Switzerland, Canton of Berne, 1511 m a.s.l., *Picea abies* forest, 25 Nov. 2012	46°35′28.3″N, 07°20′26.9″E	8
B. fuscescens	19000, 19035	Finland, Prov. Etelä-Häme, Liesjärvi, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012	60°40′17.0″N, 23°51′10.4″E	2
B. implexa	19037	Switzerland, Canton of Berne, 1511 m a.s.l., *Picea abies* forest, 25 Nov. 2012	46°35′28.3″N, 07°20′26.9″E	1
B. implexa	19038–19042	Finland, Prov. Etelä-Häme, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012	60°42′04.3″N, 23°54′41.9″E	3
B. implexa	19043–19045	Finland, Prov. Etelä-Häme, Liesjärvi, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012	60°40′17.0″N, 23°51′10.4″E	5

[a]Vouchers deposited at Lichens Herbarium of the Universidad Complutense de Madrid (MAF-Lich).

APPENDIX 2. Percentage of successful amplification between species of *Bryoria* sect. *Implexae*, and between compared regions.

Group	*n*	*p*	Bi01	Bi02	Bi03	Bi04	Bi05	Bi06	Bi07	Bi08	Bi09	Bi10	Bi11	Bi12	Bi13	Bi14	Bi15	Bi16	Bi18	Bi19
B. capillaris	36	0.008	100	94	100	100	92	100	100	100	97	100	100	100	100	100	22	100	100	100
B. fuscescens	37	0.81	100	68	100	100	100	100	100	100	51	100	100	100	100	100	100	100	100	100
B. implexa	9	0.99	100	89	100	100	100	100	100	100	67	100	100	100	100	100	78	100	89	100
Spain	31	0.97	100	65	100	100	100	100	100	100	52	100	100	100	100	100	90	100	100	100
Switzerland	35	0.86	100	97	100	100	91	100	100	100	94	100	100	100	100	100	43	100	97	100
Finland	16	0.39	100	81	100	100	100	100	100	100	69	100	100	100	100	100	56	100	100	100
Total	82		100	81–84	100	100	97	100	100	100	72	100	100	100	100	100	63–67	100	96–99	100

Note: *n* = total number of samples analyzed; *p* = probability (according to chi-square test) that each group will equally amplify with all markers.

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Molecular studies reveal a new species of *Bryoria* in Chile

Carlos G. BOLUDA, Pradeep K. DIVAKAR, David L. HAWKSWORTH, Johana VILLAGRA and Víctor J. RICO

Abstract: *Bryoria araucana* sp. nov. is described from Chile on the basis of morphological, chemical and molecular data. It has a grey to dark greyish brown pendent thallus with the base usually black, branching angles mainly obtuse, terminal branches with few lateral branchlets acutely inserted, fumarprotocetraric acid, and often protocetraric and confumarprotocetraric acids. It is morphologically similar to the Northern Hemisphere *B. trichodes*, but lacks soralia and has inconspicuous concolorous or slightly darker pseudocyphellae. *Bryoria glabra* is also reported for the first time from the Southern Hemisphere. New phylogenetic data based on ITS, mtSSU and *MCM7* analyses suggest that *Bryoria* sect. *Bryoria* is polyphyletic and needs revision.

Key words: Conguillío National Park, lichen, *Parmeliaceae*, phylogeny, taxonomy

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Introduction

Bryoria Brodo & D. Hawksw. is the largest genus in the alectorioid clade of the family *Parmeliaceae* (Divakar *et al.* 2015), which inhabits temperate to alpine regions worldwide. It has been comprehensively studied in North America and northern Europe (Brodo & Hawksworth 1977; Myllys *et al.* 2011*a*); however, morphological simplicity and chemical variability make its taxonomy difficult, and recent molecular data have resulted in several changes (Velmala *et al.* 2009, 2014; Myllys *et al.* 2014). Recent studies have discovered additional new species in *Bryoria* from east-central Asia (Myllys *et al.* 2011*b*; Jørgensen *et al.* 2012), southern South America and the Antarctic (Olech & Bystrek 2004; Fryday & Øvstedal 2012). Temperate South America is a region with an unexpectedly low reported *Bryoria* diversity, suggesting that additional species may be awaiting discovery in the region. In the course of lichen research by one of us (JV) in the Conguillío National Park in Chile, samples of *Bryoria* growing on *Araucaria araucana* trees were collected. Molecular, morphological and chemical analyses of the specimens revealed the presence of two species: *Bryoria glabra* (Motyka) Brodo & D. Hawksw. and another that did not group with any known species in our ongoing morphological, chemical, and phylogenetic analyses. This second species is therefore described here as new.

C. G. Boluda, P. K. Divakar, D. L. Hawksworth, J. Villagra and V. J. Rico: Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense, Plaza de Ramón y Cajal s/n, Madrid 28040, Spain. Email: carlos.g.boluda@gmail.com

D. L. Hawksworth is also at: Department of Life Sciences, The Natural History Museum, Cromwell Road, London SW7 5BD, UK; and Comparative Plant and Fungal Biology, Royal Botanic Gardens, Kew, Surrey TW9 3DS, UK.

Materials and Methods

The specimens collected were analyzed morphologically using standard methods (Smith *et al.* 2009) using a Nikon SMZ-1000 stereomicroscope and a Nikon Eclipse-80i microscope, and photographs were taken with a Nikon 105 mm f/2.8D AF Micro-Nikkor lens coupled to a Nikon D90 camera. Spot tests with C, K, KC, and Pd were carried out as explained in Brodo & Hawksworth (1977). For thin-layer chromatography (TLC), solvents A, B and C were used to run concentrated lichen extracts in 50 °C acetone spotted onto silica gel 60 F_{254} aluminium sheets (Merck, Darmstadt), according to standard methods (Orange *et al.* 2010).

TABLE 1. *Specimen information and GenBank accession numbers for the taxa used in this study. Newly obtained sequences are in bold.*

Taxon name	Specimen number	Locality	Chemistry	GenBank accession numbers		
				ITS	mtSSU	MCM7
Bryoria americana	1	Finland, Kainuu	Fum	HQ402677	HQ402636	KJ948017
B. americana	2	Canada, B. C.	Fum, Cfum, Pro	HQ402678	HQ402637	KJ948016
B. araucana	1	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975402**	**KP939085**	**KP975410**
B. araucana (holotype)	2	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975405**	**KP939082**	**KP975413**
B. araucana	3	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975404**	**KP939083**	**KP975412**
B. araucana	4	Chile, La Araucaría IX R.	Fum, Cfum,	**KP975403**	**KP939084**	**KP975411**
B. araucana	5	Chile, La Araucaría IX R.	Fum, Cfum	**KP975407**	**KP939081**	**KP975414**
B. araucana	6	Chile, La Araucaría IX R.	Fum, Pro, Cfum,	**KP975406**	**KP939080**	**KP975415**
B. bicolor	1	Finland, Etelä-Häme	-	HQ402691	HQ402645	KJ948018
B. bicolor	2	Finland, Koillismaa	Bar, Pso, Fum	HQ402689	HQ402644	KJ948019
B. confusa		China, Yunnan	-	HQ402686	-	KJ948024
B. divergescens		China, Yunnan	Fum, Pro, Cfum, Qua	HQ402705	HQ402654	KJ948025
B. fastigiata		China, Yunnan	Fum, Pro, Cfum	HQ402706	HQ402655	-
B. fremontii	1	Canada, B. C.	No subs	FJ668503	FJ668436	KJ948028
B. fremontii	2	Finland, Koillismaa	Vul in soralia.	FJ668498	FJ668432	KJ948029
B. furcellata	1	Finland, Etelä-Savo	Fum, Pro, Cfum	HQ402722	HQ402667	KJ948031
B. furcellata	2	Canada, Manitoba	Fum, Pro, Cfum	HQ402721	HQ402666	KJ948030
B. fuscescens	1	Finland, Koillismaa	Fum, Pro, Cfum	GQ996291	GQ996332	KJ948035
B. fuscescens	2	Finland, Åland	Fum, Pro, Cfum	GQ996290	GQ996322	KJ948032
B. glabra	1	Canada, B. C.	Fum in soralia.	HQ402728	HQ402673	KJ948037
B. glabra	2	Finland, Koillismaa	Fum in soralia.	FJ668494	FJ668428	KJ948036
B. glabra	7	Chile, La Araucaría IX R.	Fum	**KP975408**	**KP939086**	**KP975417**
B. hengduanensis		China, Yunnan	Usn, Fum, Pro, Cfum	HQ402704	HQ402653	KJ948038
B. lactinea		China, Yunnan	Fum, Pro, Cfum	HQ402699	-	KJ948050
B. nadvornikiana	1	Finland, Kainuu	Bar, Ale, Fum, Cfum, Atr	HQ402718	HQ402663	KJ948053
B. nadvornikiana	2	Iran, East-Azarbaijan	Bar	HQ402720	HQ402665	KJ948052
B. nitidula	1	Sweden, Ångermanland	-	HQ402713	HQ402658	KJ948054
B. nitidula	2	Greenland	Fum, Pro, Cfum	HQ402711	HQ402656	KJ948055
B. poeltii		China, Yunnan	Fum	HQ402701	HQ402650	KJ948057
B. simplicior	1	Finland, Koillismaa	Fatty acids	HQ402714	HQ402659	KJ948063
B. simplicior	2	Russia, Sakha Republic	No subs	HQ402716	HQ402661	KJ948062
B. simplicior	3	Norway, Troms	No subs	**KP975409**	-	**KP975416**
B. smithii	1	Finland, Varsinais-Suomi	No subs	HQ402684	HQ402642	KJ948065
B. smithii	2	India, Uttaranchal	-	HQ402685	HQ402643	KJ948064
B. tenuis	1	Finland, Kainuu	Fum	HQ402694	HQ402648	KJ948074
B. tenuis	2	Sweden, Dalarna	Fum	HQ402695	HQ402649	KJ948073
B. trichodes	1	Canada, Newfoundland	Fum, Cfum, Pro, Atr	HQ402710	-	KJ948075
B. trichodes	2	Russia, Kamchatka	-	KJ947952	-	KJ948076
Pseudephebe pubescens		USA, Alaska	No subs	HQ402676	HQ402635	KJ948091

Chemistry as follows: Ale = alectorialic acid, Atr = atranorin, Bar = barbatolic acid, Cfum = confumarprotocetraric acid, Fum = fumarprotocetraric acid, Gyr = gyrophoric acid, Nor = norstictic acid, Pro = protocetraric acid, Pso = psoromic acid, Qua = quaesitic acid, Usn = usnic acid, Vul = vulpinic acid, No subs = no lichen substances detected.

For the best resolution in solvent C, the spotted plate was left to stand for 10 min before running in an acetic acid atmosphere.

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Barcelona) with a slight modification to the manufacturer's instructions (Crespo *et al.* 2001; Divakar *et al.* 2012). Three loci were amplified: 1) nrITS, with ITS1FKYO2 (5'-TAG AGG AAG TAA AAG TCG TAA-3') and ITS4KYO2 (5'-RBT TTC TTT TCC TCC GCT-3'; Toju *et al.* 2012) primers; 2) mSSU rDNA, with mtSSU1 (5'-AGC AGT GAG GAA TAT TGG TC-3') and mtSSU3R (5'-ATG TGG CAC GTC TAT AGC CC-3'; Zoller *et al.* 1999) primers; and 3) the low copy protein coding gene *MCM7*, with MCM71348rev (5'-GAY TTD GCI ACI CCI GGR TCW CCC AT-3') and MCM7-709f (5'-ACI MGI GTI TCV GAY GTH AAR CC-3'; Schmitt *et al.* 2009) primers. For amplification, a reaction mixture of

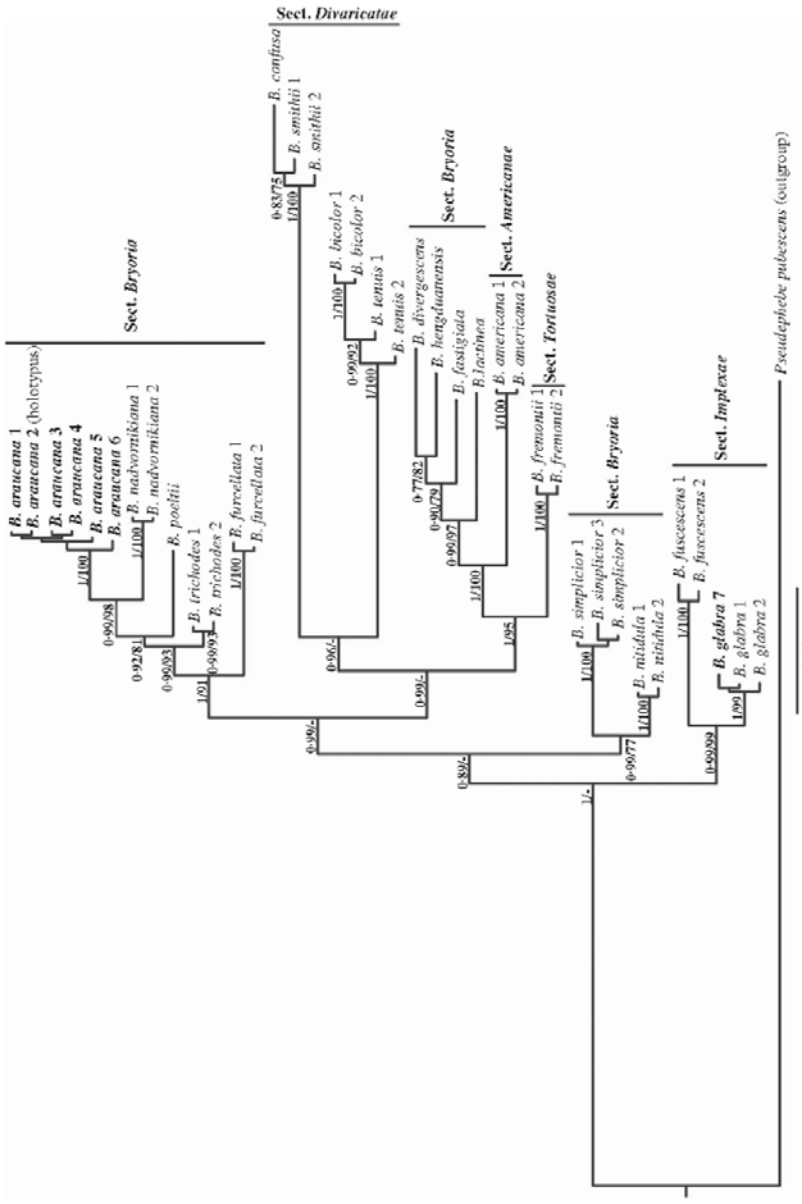

FIG. 1. Phylogenetic relationships of *Bryoria* species used in this study, 38 samples representing 19 species, based on ITS, mtSSU, and *MCM7* markers analyzed in a concatenated data matrix. Tree topology depicts the results of the Bayesian Markov chain Monte Carlo (B/MCMC) analysis. Posterior probabilities and bootstrap values, when coincident with the Bayesian tree, are given on the node branches. Sections according to Myllys *et al.* (2011b). *B. glabra* (bold) = Chilean specimen. *B. araucana* (bold) = new species. *Pseudephebe pubescens* used as outgroup.

25 µl was used containing 18 µl sterile water, 2·5 µl ×10 buffer with 2 mM $MgCl_2$, 0·5 µl dNTPs (10 mM of each base), 1·25 µl of each primer at 10 µM, 0·625 µl of DNA polymerase (1U μl^{-1}), and 1–2 µl DNA template. In failed samples the PCR was repeated using PuReTaq Ready-To-Go PCR Beads (2·5 U of PuReTaq DNA Polymerase, 200 µM of each dNTP, BSA, buffer reaction and stabilizers: 10 mM Tris-HCl ph 9·0, 50 mM KCl, 1·5 mM $MgCl_2$; GE Healthcare, Little Chalfont, UK) adding to the lyophilized bead 20 µl of sterile water, 1 µl of each primer at 10 µM, and 1·5 µl of DNA template.

Amplifications were run in an automatic thermocycler (XP Cycler, Bioer, Hangzhou) using the following parameters for ITS rDNA and mtSSU rDNA: initial denaturation 5 min at 95 °C, then 35 cycles of 1 min at 95 °C, 1 min at 56 °C, 1·5 min at 72 °C, and a final extension of 10 min at 72 °C. For *MCM7* we used a touchdown cycling process: initial denaturation 10 min at 94 °C, then followed by 4 cycles of 45 s at 94 °C, 50 s at 56 °C, 1 min at 72 °C; 4 cycles of 45 s at 94 °C, 50 s at 54 °C, 1 min at 72 °C; 36 cycles of 45 s at 94 °C, 50 s at 52 °C, 1 min at 72 °C and a final extension of 8 min at 72 °C. PCR products were cleaned using illustra™ ExoProStar (GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. Sequencing was performed by the Unidad de Genómica (Parque Científico de Madrid).

DNA sequences obtained were manually adjusted using SeqMan version 7.0 (DNAstar, Madison) and MEGA5 (Tamura *et al.* 2011). Some GenBank sequences from Myllys *et al.* (2011*b*; Table 1) were added to the file and aligned using MAFFT version 7 (Katoh & Standley 2013), with the G-INS-I alignment algorithm, a scoring matrix of 1 PAM/k = 2, and 0·1 as offset value. Gblocks version 0.91b (Castresana 2000) was used to delete non-conserved GAPs, allowing smaller final blocks, gap positions within the final blocks, and less strict flanking positions. The alignments of each region and the concatenated one were analyzed using maximum likelihood (ML) and Bayesian (B/MCMC) approaches, with *Pseudephebe pubescens* as outgroup to root the tree (Divakar *et al.* 2015). For maximum likelihood (ML) tree reconstruction, the program RAxML v7.2.8 (Stamatakis 2006) implemented on the Cipres Science Gateway (Miller *et al.* 2010) was used. We selected the GTRGAMMA model, which includes a parameter (Γ) for rate heterogeneity among sites and chose not to include a parameter to estimate the proportion of invariable sites (Stamatakis 2006; Stamatakis *et al.* 2008). Support values were assessed using the 'rapid bootstrapping' option with 1000 replicates. For Bayesian reconstruction, MrBayes v3.2.1 (Ronquist & Huelsenbeck 2003) was used, assuming the general time reversible model (Rodriguez *et al.* 1990) and a discrete gamma distribution with six rate categories (GTR + G). The nucleotide-substitution model and parameters were selected using the Akaike Information Criterion as implemented in jModelTest (Posada 2008). A run with four million generations, starting with a random tree and employing eight simultaneous chains, was executed. Every 400th tree was saved to a file. We plotted the log-likelihood scores of sample points against generations using TRACER v1.5 (http://beast.bio.ed.ac.uk/Tracer) and determined that stationarity had been achieved when the log-likelihood values of the sample points reached an equilibrium value (Huelsenbeck & Ronquist 2001), discarding the trees obtained before stationarity was reached. Posterior probabilities (PPs) were obtained from the 50% majority-rule consensus of sampled trees after excluding the initial 25% as burn-in. The phylogenetic trees were drawn using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree).

Results and Discussion

The tree obtained from the concatenated ITS, mtSSU and *MCM7* dataset (Fig. 1) is mainly based on sequences published by Myllys *et al.* (2011*b*), who performed a parsimony analysis obtaining five infrageneric sections. Here we subjected those sequences to maximum likelihood and Bayesian analyses, resulting in a different and better supported tree topology. This discrepancy may not be due to the phylogenetic reconstruction method, but to different sampling and the loci used. Sections *Americanae*, *Divaricatae*, *Implexae* and *Tortuosae* are resolved as monophyletic, but in different tree locations than those of Myllys *et al.* (2011*b*). Section *Implexae* appears as basal rather than derived, and sections *Americanae* and *Tortuosae* are no longer basal. Section *Divaricatae* seems justified, but section *Bryoria* was recovered as polyphyletic and split into three monophyletic groups. In view of this, sections *Americanae*, *Tortuosae* (with one sequenced species each) and *Bryoria* (polyphyletic) will evidently need revision after more detailed analysis has been undertaken. At the species level, *Bryoria tenuis* appears paraphyletic with *B. bicolor*, and *B. smithii* paraphyletic with *B. confusa*, but due to the small number of specimens included in this study it would be premature to propose any change here.

Analyses of the Chilean specimens (Table 1; Fig. 1) revealed the presence of *Bryoria glabra*, the first record from the Southern Hemisphere, and a set of different specimens that did not group with any known species. These were phylogenetically close to the Northern Hemisphere *Bryoria nadvornikiana* (Gyeln.) Brodo & D. Hawksw. but they contained fumarprotocetraric rather than barbatolic acid as the

FIG. 2. *Bryoria araucana*, holotype. A, habitat; B, habit; C, detail of branching pattern; D & E, detail of pseudocyphellae. Scales: B = 1 cm; C = 1 mm; D = 0·15 mm; E = 0·25 mm.

main substance. The Chilean specimens were quite different from *B. nadvornikiana* morphologically in that they lacked extensive blackened bases and short, perpendicular, lateral, spinule-like branches. Additionally, they were morphologically and chemically similar to the Northern Hemisphere *Bryoria trichodes* (Michx.) Brodo & D. Hawksw. but lacked soralia, although soralia are not found in every specimen of *B. trichodes*.

The material is therefore described here as a new species.

The Species

Bryoria araucana Boluda, D. Hawksw. & V. J. Rico sp. nov.

MycoBank No.: MB811960

Resembles the Northern Hemisphere circumboreal *Bryoria trichodes*, but is distinct molecularly, without soralia, and with less conspicuous pseudocyphellae.

Type: Chile, IX Región de La Araucanía, Provincia de Cautín, Comuna de Melipeuco, Conguillío National Park, Tramo Contrabandistas, Sendero Las Araucarias, close to Conguillío Lake, 38°39'13·57"S, 71° 37'05·27"W, 1215 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 31 August 2014, *J. Villagra* 2 (MAF-Lich. 19718—holotype). GenBank accession numbers: KP975405 (ITS), KP939082 (mtSSU), and KP975413 (MCM7).

(Fig. 2)

Thallus pendent to subpendent, 6–12 cm long; isotomic to anisotomic dichotomously branched, angles between dichotomies mainly obtuse, rarely acute; branches terete, even, main branches at base 0·2–0·4 mm diam., tips to 0·1 mm diam.; terminal portions with few lateral branchlets acutely inserted. *Surface* dark grey to dark greyish brown, shiny, base ordinarily black; cortex prosoplectenchymatous. *Soralia* and *isidia* lacking. *Pseudocyphellae* inconspicuous, depressed, fusiform, concolorous to slightly darker than the thallus, sometimes faintly pruinose, straight or twisted, up to 1·5 mm long. *Photobiont* trebouxioid.

Apothecia and *conidiomata* unknown.

Chemistry. Inner cortex and medulla C−, K−, KC−, PD+ yellow turning red, sometimes faint. TLC: fumarprotocetraric acid as the main substance, with protocetraric and confumarprotocetraric acids in trace amounts.

Etymology. Named after the IX Región de la Araucanía in Chile, which is the only known area for the species, as was the case in the name *Araucaria araucana*.

Distribution and ecology. Known only from the type locality and immediate surroundings in the Parque Nacional Conguillío, IX Región de La Araucanía (Chile), occurring on trunks of *Araucaria araucana* in mature open forests (Fig. 2A). Those forests are characteristic of the upper supratemperate bioclimatic belt with the ultraperhumid rainfall regime of the South American Temperate Region (Amigo & Ramírez 1998). Furthermore, the mean annual precipitation in the area, which falls mainly as snow, is *c.* 2000 mmy^{-1}, and the mean annual temperature is 8·6 °C, with dry and hot short summers (Di Castri & Hajek 1976). *Bryoria araucana* is more frequent on the north-facing trunks exposed to humid winds, growing with *Coelopogon epiphorellus*, *Protousnea dusenii*, *P. magellanica*, *P. poeppigii*, and *Platismatia glauca*. On the south-facing sides of the trunks it is less frequent, growing with *Nephroma antarcticum*, *Pseudocyphellaria coriifolia*, *P. flavicans*, and *P. granulata*. It may be anticipated that *B. araucana* will be found to have a wider distribution in the temperate forests of the Southern Hemisphere that are almost unexplored for alectorioid lichens.

Conservation status. Although the new species seems not to be frequent, it occurs in a protected area (Parque Nacional Conguillío, Chile). No special actions to conserve the species are currently required.

Additional specimens examined. Bryoria araucana **Chile:** *IX Región de La Araucanía, Provincia de Cautín*: Comuna de Melipeuco, Parque Nacional Conguillío, Tramo Contrabandistas, Sendero Las Araucarias, close to Conguillío Lake, 38°39'14·83"S, 71°37'01·06"W, 1211 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 2013, *J. Villagra* 5 & 6 (MAF-Lich. 19723, 19724); *ibid.*, 38°39'13·57"S, 71°37'05·27"W, 1215 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 2014, *J. Villagra* 1, 3 & 4 (MAF-Lich. 19719, 19720, 19721).

Bryoria glabra **Chile:** *IX Región de La Araucanía, Provincia de Cautín*: Comuna de Melipeuco, Parque Nacional Conguillío, Tramo Contrabandistas, Sendero Las Araucarias, close to Conguillío Lake, 38°39'13·57"S, 71°37'05·27"W, 1215 m, *Araucaria araucana* forest, on the north side of an araucaria trunk, 2014, *J. Villagra* 7 (MAF-Lich. 19722).

Bryoria araucana and the Northern Hemisphere species *B. trichodes* form divergent independent clades which are well supported

TABLE 2. *Comparison of the chemistry, main morphological characters and distribution of five phylogenetically related species of* Bryoria *including* B. araucana *based on our observations and bibliographic references (Brodo & Hawksworth 1977; Bystrek 1969; Myllys* et al. *2011a; Wang & Chen 1994).*

	B. araucana	*B. nadvornikiana*	*B. poeltii*	*B. trichodes*	*B. furcellata*
Main chemistry	Fum	Bar, Ale, ± Atr, Fum in soralia	Fum	Fum, Chlor	Fum
Thallus	Pendent	Caespitose (base) to pendent	Caespitose (base) to pendent	Pendent	Caespitose
Pseudocyphellae	Inconspicuous, dark grey-brown	Inconspicuous, white	Conspicuous, dark brown-black	Conspicuous, white to brownish	Absent
Soralia	Absent	Tuberculate to fissural, white	Tuberculate to fissural, dark, spinulose	Rare, fissural, white	Fissural, white, spinulose (tufts)
Spinules or spinulose branches	On terminal portions, sparse	Lateral, sparse to frequent	Sparse, also on soralia	Lateral, sparse	Sparse to frequent
Colour	Dark grey-brown, base usually darker	Pale to dark brown-violet, base generally black	Dark brown to black	Pale to dark brown	Pale to dark brown, base often darker
Distribution	Chile, South America	Europe, Africa, Asia, Hawaii, North America	Himalayas	Asia, North America	Europe, Macaronesia, Asia, Oceania, North and Central America

Ale = Alectorialic acid, Atr = Atranorin, Bar = Barbatolic acid, Chlor = Chloratranorin, Fum = Fumarprotocetraric acid.

(Fig. 1). The two species are very similar in morphology and chemistry (cf. Brodo & Hawksworth 1977), but *B. araucana* develops less conspicuous pseudocyphellae, lacks atranorin, and apothecia and soralia are unknown. *Bryoria nadvornikiana*, *B. poeltii* (Bystrek) Brodo & D. Hawksw. and *B. furcellata* (Fr.) Brodo & D. Hawksw. are phylogenetically related species, but they can be distinguished by the characters shown in Table 2. Based on the molecular results, restricted distribution, development of inconspicuous pseudocyphellae, and absence of soralia, the new species is well supported.

The *Bryoria glabra* specimen appears to be the first record from the Southern Hemisphere (Fig. 1; Table 1). It is characterized by repeatedly oval, whitish soralia and regular branching, with rounded and obtuse angles between the branches, and contains fumarprotocetraric acid (Brodo & Hawksworth 1977; Myllys *et al.* 2011*b*).

Five additional *Bryoria* species are reported in the literature from southern South America (Argentina and Chile): *Bryoria bicolor* (Ehrh.) Brodo & D. Hawksw. (Calvelo & Liberatore 2002), *B. chalybeiformis* (L.) Brodo & D. Hawksw., *B. mariensis* Øvstedal *et al.* (Fryday & Øvstedal 2012), *B. implexa* (Hoffm.) Brodo & D. Hawksw., and *B. austromontana* P. M. Jørg. & D. J. Galloway (Øvstedal & Lewis Smith 2004). *Bryoria araucana* is distinguished from all these species by the corticolous pendent habit, lack of soralia, branches 0·2–0·4 mm diam., and the sometimes dark basal parts. However, we consider some of these literature records dubious, and in need of verification through molecular analyses.

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Microchemical and molecular investigations reveal *Pseudephebe* species as cryptic with an environmentally modified morphology

Carlos G. BOLUDA, David L. HAWKSWORTH, Pradeep K. DIVAKAR, Ana CRESPO and Víctor J. RICO

Abstract: The results of the first molecular phylogenetic study of *Pseudephebe* are presented; a three-locus phylogeny. The genus is confirmed as monophyletic within the alectorioid clade of *Parmeliaceae*. Two major clades were recovered, which can be assigned to the two traditional taxa, *P. minuscula* and *P. pubescens*, with modifications of the species delimitation, especially the variable *P. minuscula*. These species are cryptic and cannot be confidently distinguished morphologically due to phenotypic convergence. Therefore, the use of *P. pubescens* aggr. is recommended for samples not molecularly analyzed. Contrary to previous studies, specimens of both species might have indistinct pseudocyphellae and also contain lichen substances; norstictic acid was detected in *c.* 60% of specimens tested. An SSU 1516 Group I intron is usually present in *P. minuscula* but always absent in *P. pubescens*. The species-level nomenclature is summarized and sequenced reference specimens (RefSpec) for both *Pseudephebe* species are selected. Sequences from *Bryoria mariensis* established that this name was a synonym of *P. minuscula*.

Key words: Alectorioid clade, barcoding, *Bryoria*, intron, lichen, *Parmeliaceae*, phenotypic convergence

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Introduction

Pseudephebe M. Choisy is a genus of lichenized fungi in the alectorioid clade of *Parmeliaceae* (Divakar *et al.* 2015). It is distinguished from the two other genera in the clade with non-septate colourless ascospores, *Bryoria* Brodo & D. Hawksw. and *Nodobryoria* Common & Brodo, primarily by a distinct superficial layer of cells on the cortex (Brodo & Hawksworth 1977). *Pseudephebe* species form small, fruticose to subcrustose cushion-like thalli on hard siliceous rock surfaces and have never been described as producing extrolites (i.e. secondary metabolites). The genus is known from both hemispheres, is circumpolar, and is found in mountains with arctic-alpine conditions, from Europe, North America and southern South America (Øvstedal & Smith 2001). It is, however, not known from Africa and is uncommon in Asia and Australia (Kantvilas 1994; Wang & McCune 2010). The genus traditionally comprised two morphologically similar species (Hillmann 1936; Lamb 1964; Brodo & Hawksworth 1977): 1) *P. minuscula* (Arnold) Brodo & D. Hawksw., with strongly appressed and tending to be flattened branches, the tips becoming adnate, and with internodes to 1 mm in length; and 2) *P. pubescens* (L.) M. Choisy, with terete and never strongly appressed branches, the tips generally free, and internodes 1–3 mm in length. The known distributions of the two species overlap, but *P. minuscula* is generally reported from more extreme arctic-alpine habitats than *P. pubescens*, which is usually more common in moister areas (Myllys *et al.* 2011). Both species have a variable morphology, which has led

C. G. Boluda (corresponding author), D. L. Hawksworth, P. K. Divakar, A. Crespo and V. J. Rico Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense, Plaza de Ramón y Cajal s/n, Madrid 28040, Spain. Email: carlgala@ucm.es
D. L. Hawksworth is also at: Department of Life Sciences, The Natural History Museum, Cromwell Road, London SW7 5BD, UK; and Comparative Plant and Fungal Biology, Royal Botanic Gardens, Kew, Surrey TW9 3DS, UK.

to the description of many infraspecific taxa, especially forms. These forms have been delimited mainly based on branching and appression degree and the thalli become almost subcrustose in part when growing in the most severe environments. Intermediate morphs occur, particularly in extreme habitats, making morphological species delimitation difficult (Imshaug 1957; Brodo & Hawksworth 1977).

Bryoria mariensis, recently described from the Falkland Islands (Fryday & Øvstedal 2012), morphologically resembles large specimens of *P. pubescens*. It was described as containing lichenan and having a morphologically similar cortex to *Pseudephebe*; a prosoplechtenchymatous hyphal inner layer and a pseudoparenchymatous surface with knobby outermost cells. The presence of lichenan excludes a placement in *Nodobryoria* (Common 1991; Common & Brodo 1995). Furthermore, species of *Nodobryoria* also have matt rather than shiny thalli and a knobbly cortex with a jigsaw pattern in surface view. The species was described in *Bryoria*, despite the similarities in cortical structure to *Pseudephebe* species, because of the presence of pseudocyphellae, production of norstictic acid and what were referred to as soralia; all characters not previously reported in *Pseudephebe* (Fryday & Øvstedal 2012). Given the similarities between *B. mariensis* and *Pseudephebe*, we also included this species in our study.

For this study we performed morphological, microchemical, and three-locus phylogenetic analyses of a range of *Pseudephebe* specimens, and two recently collected *B. mariensis* samples from the type locality.

Materials and Methods

Materials

We studied over 120 *Pseudephebe* specimens from various institutional collections (AAS, BM, K, MAF, MSC, NMW, UBC, and ZT), and the private collection of Nastassja Noell (Reno, Nevada, USA; as hb. N. Noell). Thirty-seven of these were used for phylogenetic reconstruction, with the addition of 25 outgroup specimens (Table 1). Material that was morphologically and microchemically studied, but not molecularly analyzed, is listed according to the names used in the original identification, in Supplementary Material S1 (available online).

Morphology and chemistry

Traditional and additional characters used to distinguish *Pseudephebe pubescens* and *P. minuscula* were examined in all material studied (Table 1, cited under Taxonomy, and in Supplementary Material S1 & S2, available online). For loaned specimens the envelope identification was maintained, while new collections were identified using Brodo & Hawksworth (1977), Myllys *et al.* (2011) and Smith *et al.* (2009). Specimens were examined morphologically under a Nikon SMZ-1000 stereomicroscope, and hand-cut sections were studied using a Nikon Eclipse-80i microscope. Photographs were taken with a Nikon 105 mm f/2.8D AF Micro-Nikkor lens connected to a Nikon D90 camera. Spot tests (K, C, and PD) and thin-layer chromatography (TLC) were carried out following Orange *et al.* (2010). We used TLC solvent system C (200 ml toluene / 30 ml acetic acid), with concentrated acetone extracts at 50 °C spotted onto silica gel 60 F254 aluminium sheets (Merck, Darmstadt). The aluminium sheets were dried for 10 min in an acetic acid atmosphere to maximize resolution. The same lichen fragment used for TLC was used for DNA extraction.

Molecular techniques

DNA was extracted from a single, clean (under a dissecting microscope) lichen branch using the DNeasy Plant Mini Kit (Qiagen, Barcelona) with a slight modification to the manufacturer's instructions (Crespo *et al.* 2001). The fungal nuclear internal transcribed spacer (ITS) rDNA, and a partial sequence of the low copy protein coding genes RNA polymerase II largest subunit (*RPB1*), and minichromosome maintenance complex component 7 (*Mcm7*) were amplified using, respectively, the primers ITS1F-KYO2 (5'-TAG AGG AAG TAA AAG TCG TAA-3') and ITS4KYO2 (5'-RBT TTC TTT TCC TCC GCT-3') (Toju *et al.* 2012), RPB1 MH F (5'-ACGTCGCCGA GACCCHAARA-3'; Leavitt *et al.* 2012) and fRPB1-C rev (5'-CCNGCDATNTCRTTRTCCATRTA-3'; Matheny *et al.* 2002), and Xmcm7 F1 (5'-CGTACACYTGT GATCGATGTG-3'; Leavitt *et al.* 2011) and Mcm7-1348rev (5'-GAYTTDGCIACICCIGGRTCWC CCAT-3'; Schmitt *et al.* 2009). For amplification, we used a reaction mixture of *c.* 25 µl, containing 18 µl of sterile water, 2·5 µl of 10× buffer with 2 mM $MgCl_2$, 0·5 µl dNTPs (10 mM of each base), 1·25 µl of each primer at 10 µM, 0·625 µl of DNA polymerase (1U μl^{-1}), and 0·5–2 µl of DNA elution 2 template. For any failed samples the PCR was repeated using PuReTaq Ready-To-Go PCR Beads (2·5 U of PuReTaq DNA Polymerase, 200 µM of each dNTP, BSA, buffer reaction and stabilizers: 10 mM Tris-HCl pH 9·0, 50 mM KCl, 1·5 mM $MgCl_2$; GE Healthcare, Little Chalfont, UK), adding to the lyophilized bead 20 µl of sterile water, 1 µl of each primer at 10 µM and 2 µl of elution 1 DNA template.

Amplifications were run in an automatic thermocycler (XP Cycler, Bioer, Hangzhou, China) using the following parameters: initial denaturation of 5 min at 95 °C, then 35 cycles of 60 s at 95 °C, 60 s at 56 °C,

TABLE 1. *GenBank accession numbers and species data for specimens used for the phylogenetic tree shown in Fig. 1. Newly obtained sequences are in bold. DNAcode refers to the genetic material extracting code, newly obtained extractions preserved in MAF Herbarium DNA-bank.* Pseudephebe *species names are according to the original identifications. Specimens 4591 and 4670 have ITS intrathalline variability, and the alleles with an intron are indicated with (I).*

				GenBank Accession Numbers		
Species	Locality	Voucher specimen	DNAcode	ITS	*RPB1*	*Mcm7*
Alectoria ochroleuca	Austria, Styria	*Wedin* VIII-1998 (UPS)	MWE 1998	DQ979997	**KU668501**	**KU668455**
A. ochroleuca	Chile, Magallanes	MAF-Lich. 18296	3836	**KU647282**	**KU668502**	**KU668456**
A. sarmentosa	Sweden, Västerbotten	*Wedin* 6350 (UPS)	6350	DQ979998	DQ923678	KR995525
A. sarmentosa	Spain, Asturias	MAF-Lich. 17914	3710	**KU647283**	**KU668503**	**KU668457**
Allantoparmelia alpicola	Sweden, Lycksele Lappmark	*Wedin* 7159 (UPS)	MWE 7159	DQ979999	DQ923679	KR995527
Bryocaulon divergens	Sweden, Härjedalen	*Odelvik* 9145 (S)	MWE 158	**KU647284**	**KU668504**	**KU668458**
B. pseudosatoanum	Japan, Honshu	TNS s. n.	YO 8255	KR995272	KR995448	KR995536
B. satoanum	Japan, Honshu	*G. Thor* 28135	MWE 163	**KU647285**	**KU668505**	**KU668459**
Bryoria americana	Finland, Kainuu	*Velmala* 63 (H)	S69	HQ402677	**KU668540**	KJ948017
B. capillaris	Finland, Etelä-Savo	*Myllys* 485 (H)	L211	GQ996287	**KU668541**	KJ948022
B. capillaris	Finland, Etelä-Häme	*Haikonen* 22228 (H)	L141	FJ668493	**KU668547**	KJ948020
B. capillaris	Germany, Nordschwarzwald	MAF-Lich. 20111	3879	**KU647286**	**KU668543**	**KU668460**
B. fremontii	Spain, Asturias	MAF-Lich. 18136	3610	**KU647287**	**KU668554**	**KU668461**
B. fremontii	Finland, Etelä-Pohjanmaa	*Myllys* 490 (H)	L214	FJ668507	**KU668553**	**KU668462**
B. fuscescens	Finland, Oulun-Pohjanmaa	*Halonen* s. n. (OULU)	L189	GQ996305	**KU668548**	KJ948071
B. fuscescens	Finland, Koillismaa	*Velmala* 51 *&* *Halonen* (H)	S56	GQ996291	**KU668544**	KJ948035
B. glabra	Finland, Koillismaa	*Halonen* s. n. (OULU)	L186	FJ668494	**KU668549**	KJ948036
B. implexa	Finland, Koillismaa	*Velmala et al.* 23 (H)	S22	GQ996294	**KU668542**	KJ996315
B. implexa	UK, England	*Boluda* 3855	3855	**KU647288**	**KU668546**	**KU668464**
B. implexa	Iran, East-Azarbaijan	*Sohrabi* 4656 (H)	L244a	GQ996295	**KU668545**	KJ948042
B. nadvornikiana	Finland, Kainuu	*Velmala et al.* 73 (H)	S79	HQ402718	**KU668550**	KJ948053
B. nadvornikiana	Sweden, Dalarna	*Hermansson* 14179 (UPS)	L161	HQ402719	**KU668551**	**KU668465**
B. simplicior	Finland, Koillismaa	*Velmala et al.* 30 (H)	S30b	HQ402714	**KU668552**	KJ948063
Govardia nigricans	Chile, XII Region	MAF-Lich. 18297	3837	**KU647289**	**KU668538**	**KU668466**
G. nigricans	Norway, Troms	*Wedin* 7297 (UPS)	Wedin 7297	DQ979996	**KU668539**	**KU668467**
Bryoria mariensis	Falkland Islands	NMW.C.2015.004.8	5077	**KU647290**	**KU668519**	**KU668483**
B. mariensis	Falkland Islands	*Fryday* 10925	5075	**KU647291**	**KU668516**	**KU668480**
Pseudephebe minuscula	USA, Alaska	SRP L-0008791	4339	**KU647292**	**KU668523**	**KU668487**
P. pubescens	USA, Alaska	SRP L-0008806	4338	**KU647293**	**KU668526**	**KU668489**
P. minuscula	USA, Nevada	hb. N. Noell 1442	4784	**KU647294**	**KU668535**	**KU668500**
P. pubescens	USA, California	hb. N. Noell 1581	4781	**KU647295**	**KU668536**	–
P. pubescens	USA, Montana	S F175892	4363	**KU647296**	**KU668530**	**KU668493**
P. pubescens	USA, Montana	S F144171	4362	**KU647297**	**KU668528**	**KU668491**
P. pubescens	USA, Oregon	*Hollinger* 3971	4591	**KU647298** **KX160147 (I)**	**KU668529**	**KU668492**

TABLE 1. *Continued*

				GenBank Accession Numbers		
Species	Locality	Voucher specimen	DNAcode	ITS	*RPB1*	*Mcm7*
P. pubescens	USA, Washington	hb. N. Noell 1557	4783	**KU647299**	**KU668537**	–
P. pubescens	Chile, Magallanes	MAF-Lich. 20105	5073	**KU647300**	**KU668517**	**KU668481**
P. pubescens	Chile, Magallanes	MAF-Lich. 20106	5074	**KU647301**	**KU668518**	**KU668482**
P. minuscula	Norway, South Nordland	MAF-Lich. 20107	5079	**KU647302**	**KU668533**	**KU668496**
P. minuscula	Norway, Sogn og Fjordane	MAF-Lich. 20102	5080	**KU647303**	–	**KU668497**
P. minuscula	Sweden, Jämtland	S F149958	4360	**KU647304**	**KU668527**	**KU668490**
P. minuscula	Sweden, Jämtland	S F177970	4367	**KU647305**	**KU668524**	–
P. pubescens	Sweden, Västerbotten	S F240229	4364	**KU647306**	**KU668520**	**KU668484**
P. pubescens	Austria, Tirol	MAF-Lich. 17091	4201	**KU647307**	**KU668521**	**KU668485**
P. pubescens	Romania, Hunedoara	MAF-Lich. 19475	4668	**KU647308**	**KU668522**	**KU668486**
P. minuscula	Portugal, Beira Baixa	MAF-Lich. 19472	4590	**KU647309**	**KU668525**	**KU668488**
P. minuscula	Portugal, Minho	MAF-Lich. 19473	4670	**KU647310** **KX160146 (I)**	**KU668534**	**KU668498**
P. pubescens	Spain, Asturias	MAF-Lich. 17838	4199	**KU647311**	–	**KU668499**
P. aff. *minuscula*	Spain, Segovia	MAF-Lich. 20103	5071	**KU647312**	**KU668531**	**KU668494**
P. aff. *minuscula*	Spain, Segovia	MAF-Lich. 20104	5072	**KU647313**	**KU668532**	**KU668495**
P. pubescens	Norway, Sogn og Fjordane	MAF-Lich. 20100	5081	**KU647314**	–	**KU668475**
P. pubescens	Norway, Sogn og Fjordane	MAF-Lich. 20101	5082	**KU647315**	**KU668512**	**KU668476**
P. pubescens	Norway, South Nordland	MAF-Lich. 20108	5078	**KU647316**	**KU668515**	**KU668479**
P. pubescens	Sweden, Jämtland	S F149572	4365	**KU647317**	**KU668513**	**KU668477**
P. pubescens	Switzerland, Uri	MAF-Lich. 19476	4669	**KU647318**	**KU668511**	**KU668474**
P. pubescens	Spain, Asturias	MAF-Lich. 17907	4198	**KU647319**	**KU668508**	**KU668470**
P. pubescens	Spain, Asturias	MAF-Lich. 17915	4196	**KU647320**	**KU668510**	**KU668472**
P. pubescens	Spain, Asturias	MAF-Lich. 17930	4197	**KU647321**	**KU668514**	**KU668478**
P. pubescens	Spain, Asturias	MAF-Lich. 18112	3709	**KU647322**	–	**KU668471**
P. pubescens	Spain, León	MAF-Lich. 19474	4671	**KU647323**	–	**KU668473**
P. pubescens	Spain, Teruel	MAF-Lich. 16841	4200	**KU647324**	**KU668509**	–
P. pubescens	Spain, Zamora	MAF-Lich. 19470	4589	**KU647325**	**KU668507**	**KU668469**
P. pubescens	Spain, Zamora	MAF-Lich. 19471	3919	**KU647326**	**KU668506**	**KU668468**

90 s at 72 °C, and a final extension of 10 min at 72 °C for ITS; initial denaturation of 10 min at 94 °C, followed by 4 cycles of 45 s at 94 °C, 50 s at 56 °C, 1 min at 72 °C, 4 cycles of 45 s at 94 °C, 50 s at 54 °C, 1 min at 72 °C, 36 cycles of 45 s at 94 °C, 50 s at 52 °C, 1 min at 72 °C and a final extension of 8 min at 72 °C for *RPB1* and *Mcm7*. Double bands in an electrophoresis gel of PCR products were isolated using the FavorPrep™ MicroElute Gel / PCR Purification Kit (Favorgen® Biotech, Vienna, Austria) following the manufacturer's instructions. PCR products were cleaned using Illustra™ ExoProStar (GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. Sequencing was performed by the Unidad de Genómica (Parque Científico de Madrid). The DNA sequences obtained were manually adjusted using SeqMan version 7.0 (DNAstar, Madison, USA) and MEGA5 (Tamura *et al.* 2011).

Phylogenetic analyses

Alignments for each locus were performed using MAFFT v7 (http://mafft.cbrc.jp/alignment/server/; Katoh & Standley 2013) with the G-INS-i alignment algorithm and '1PAM/K = 2' scoring matrix, with an offset value of 0·1, and the remaining parameters set as default. Some *Pseudephebe* specimens contained an intron at the 3' end of SSU rDNA, which was removed for the analysis at this level. While alignments for *RPB1* and *Mcm7* were straightforward and did not require manual corrections, exploratory analyses revealed several ambiguous regions in the ITS alignment. Therefore, we used the program Gblocks v0.91b (Talavera & Castresana 2007) to delimit and remove ambiguous alignment nucleotide positions from the final ITS alignment using the online web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) and implementing the options for a less stringent selection of ambiguous nucleotide positions, including the 'Allow smaller final blocks', 'Allow gap positions within the final blocks', and 'Allow less strict flanking positions' options. The single and concatenated datasets were analyzed using maximum likelihood (ML) and Bayesian (B/MCMC) approaches. To detect topological conflicts among loci, the CADM test (Legendre & Lapointe 2004; Campbell *et al.* 2011) was performed using the function 'CADM.global' implemented in the library 'ape' of R (Paradis *et al.* 2004). The analysis resulted in a W of 0·83, which means there is no evidence of well-supported topological conflict. For the maximum likelihood (ML) tree reconstructions, the program RAxML v7.2.8 (Stamatakis 2006) implemented on the Cipres Science Gateway (http://qball2.sdsc.edu:7070/portal2/home.action; Miller *et al.* 2010) was used with the GTRGAMMA model (Stamatakis 2006; Stamatakis *et al.* 2008). Support values were assessed using the 'rapid bootstrapping' option with 1000 replicates. For the concatenated dataset, the ITS regions ITS1, 5.8S and ITS2 were partitioned and for the protein-coding markers we used a three-partition approach, with the first, second, and third codon positions as separate model partitions. For the Bayesian reconstruction, MrBayes v3.2.1 (Ronquist & Huelsenbeck 2003) was used. Models of DNA sequence evolution for each locus were selected with the program jModeltest2.0 (Darriba *et al.* 2012), using the Akaike Information Criterion (AIC; Akaike 1974). The best-fit model of evolution was as follows: ITS, ITS1 = TIM2 + G, 5.8S = K80, ITS2 = TIM3ef + G; *RPB1*; 1st position, TIM2 + I, 2nd position, TrN, 3rd position, TIM1ef + G; *Mcm7*; 1st position, TPM3, 2nd position, F81, 3rd position, TrNef + G. A run with 10 million generations, starting with a random tree and employing 12 simultaneous chains, was executed. Every 500th tree was saved to a file. We plotted the log-likelihood scores of sample points against generations using Tracer v1.5 (Rambaut *et al.* 2014) and determined that stationarity had been achieved when the log-likelihood values of the sample points reached an equilibrium and ESS values exceeded 200 (Huelsenbeck & Ronquist 2001). Preliminary analysis resulted in an overestimation of branch lengths and to correct this we used the uniform compound Dirichlet prior 'brlenspr = unconstrained:gammadir (1,1,1,1)' (Zamora *et al.* 2015), obtaining rather reasonable branch length estimates. Posterior probabilities (PPs) were obtained from the 50% majority rule consensus of sampled trees after excluding the initial 25% as burn-in. The phylogenetic tree was drawn using FigTree v1.4 (Rambaut 2009).

Species delimitation

In order to establish species limits in the phylogenetic tree, three computational approaches were used: 1) Automatic Barcode Gap Discovery ABGD (Puillandre *et al.* 2011) based on barcode gaps using genetic distances; 2) Poison Tree Processes PTP (Zhang *et al.* 2013), based on gene trees; and 3) Bayesian Phylogenetics and Phylogeography BP&P (Yang & Rannala 2010), based on a multispecies coalescent model for species validation.

Results

Molecular analysis

The GenBank accession numbers of 146 newly obtained sequences are included in Table 1. The final concatenated matrix used as input for the phylogenetic reconstruction contained 1448 unambiguously aligned base pairs (bp) (496 bp for *RPB1*, 458 for *Mcm7* and 494 for ITS), with no topological incongruences among loci. Alignments are available in TreeBASE (TB2:S15972). The tree reconstruction (Fig. 1) included all genera belonging to the alectorioid clade (Divakar *et al.* 2015), with the exception of *Nodobryoria* as no DNA-fresh material could be obtained, and using *Allantoparmelia*

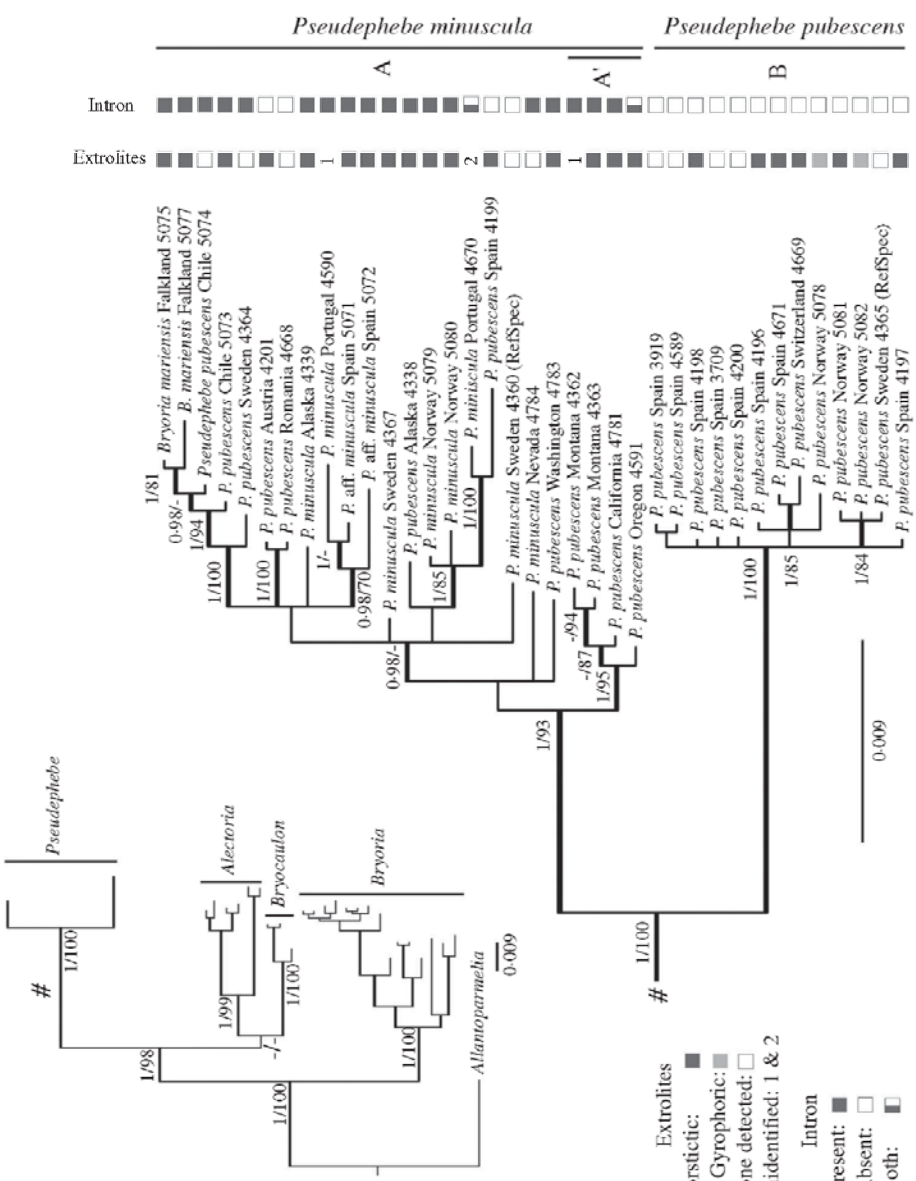

FIG. 1. Phylogenetic consensus tree based on ITS, *RPB1* and *MCM7* markers analyzed as a concatenated data matrix (Table 1). Whole tree in the upper left corner using *Allantoparmelia* as outgroup, with the *Pseudephebe* clade shown in detail. Tree topology depicts the results of Bayesian inference, showing significant posterior probabilities ≥0·95 and bootstrap values ≥70% obtained in the maximum likelihood analysis. Intron presence and extrolite composition are indicated on the lower left corner. RefSpec = sequenced reference specimen.

(Vain.) Essl. as outgroup. The genera *Alectoria* Ach. (including *Gowardia* Halonen *et al.*; see Lumbsch & Huhndorf 2010), *Bryocaulon* Kärnefelt, and *Bryoria* were included to check for any possible alternative affinities for *B. mariensis*. Several species for each genus were included to facilitate the comparison of species branch lengths with the ones in *Pseudephebe*. *Pseudephebe* was shown to be monophyletic, including *B. mariensis* within it. Two isolated clades were recovered within the genus (A and B; Fig. 1). Clade A is a variable group containing two major clades, one of which is well-supported so we distinguish it as clade A'.

The region amplified with the primers ITS1FKYO2 and ITS4KYO2 comprises the ITS (ITS1, 5.8 S and ITS2) and a small portion of the 3' end of the SSU gene, which included in some specimens a group I intron of 233 bp inserted in site 1516 relative to the SSU rDNA sequence of *Escherichia coli* (Gutiérrez *et al.* 2007) or site 1777 of *Saccharomyces cerevisiae*. This intron was detected in many but not all samples in clade A (Fig. 1), but was absent from all samples in clade B. In the electrophoresis gel of two samples, two bands corresponding to sequence lengths with and without the intron were distinguished; we checked that result by sequencing each band separately. ITS and SSU are multicopy loci and as DNA was extracted from a single branch, this result suggests that some copies could contain the intron while others would not.

Morphological and chemical analyses

Morphological and chemical characters of the specimens studied in the phylogenetic analysis are presented in Table 2. The characters traditionally used to distinguish *Pseudephebe minuscula* from *P. pubescens* (i.e. loose or dense appearance, evenly/unevenly thickened branches, branch diameter, internode branch length, and presence of flattened branches) were very variable. The gradient of values found made unequivocal separation into two groups difficult. However, some of the characters studied were taxonomically informative. The presence of a flattened branching pattern, which is related to the presence of dorsoventrally flattened branches, had fewer intermediate states than other characters. The profusion of new ramifications on branch tips (Figs 2 & 3) was also informative when used together with other characters. Characters related to apothecia and pycnidia, such as spore size and the presence of marginal cilia, however, could not be adequately assessed as only four of the *Pseudephebe* specimens analyzed molecularly had apothecia (see Supplementary Material S2, available online). On those specimens, ciliate apothecia were observed in both clades, and spore measurements were not discriminatory.

Pseudocyphellae, reported here for the first time in the genus, were observed in 23 of the genetically analyzed samples (62%). They varied from inconspicuous to very conspicuous (Fig. 3), and in two samples were perforated. The presence or absence of pseudocyphellae is a taxonomically informative character at the generic level in different clades of *Parmeliaceae* (Elix 1993; Crespo *et al.* 2010). Although reported in the original species description, no true soralia were observed in *Bryoria mariensis*, neither in the type material nor in the additional specimens studied, but frequent short side-branch hapters with globose ends were present.

Pseudephebe is described in all previous literature as lacking extrolites detectable by spot tests or TLC (Brodo & Hawksworth 1977; Smith *et al.* 2009; Myllys *et al.* 2011). However, *c.* 60% of the studied genetically samples contained norstictic acid, generally in small quantities or in traces, but in some as a major substance. In some specimens of both *P. pubescens* and *P. minuscula*, we observed the characteristic needle-like red crystals of norstictic acid after the application of K. The presence of norstictic acid was not correlated with geographical distribution or any morphological character studied. In addition, a similar spot (with TLC) to gyrophoric acid, and two unidentified substances (substances 1 and 2), were also detected in some samples.

A selection of the most informative characters that can help to distinguish the two *Pseudephebe* species recognized here (i.e. clades A and B) is provided in Table 3.

TABLE 2. *Main characters used to distinguish* Pseudephebe *species in specimens used for the phylogenetic trees shown in Fig. 1.* Pseudephebe *species names are according to the original identifications. Internode length and branch width measurements,* n = *30, are given with extreme values in brackets. RefSpec = sequenced reference specimen; ± = ambiguous.*

Clade	Species	DNAcode	Extrolites	SSU intron	Internode length (mm)	Branches width (mm)	Pseudocyphellae	Profusely branched tips	Old branches appressed	Flattened branches
A	*Bryoria mariensis*	5077	Norstictic	+	<1–7	0·26 (0·1–1·0)	+	–	–	–
A	*B. mariensis*	5075	Norstictic	+	<1–7	0·20 (0·1–0·4)	+	–	±	±
A	*Pseudephebe minuscula*	4339	Norstictic	+	<1	0·18 (0·1–0·3)	–	+	+	+
A	*P. pubescens*	4338	Norstictic	+	<1	0·18 (0·1–0·2)	+	+	–	±
A	*P. minuscula*	4784	Absent	+	<1	0·32 (0·2–0·5; crusty)	+	+	+	+
A'	*P. pubescens*	4781	Norstictic	+	>1	0·10 (0·1–0·2)	perforated	+	–	–
A'	*P. pubescens*	4363	Norstictic	+	<1–2	0·17 (0·1–0·3)	±	+	±	+
A'	*P. pubescens*	4362	Greyish pale spot	+	<1	0·17 (0·1–0·2)	–	+	±	±
A'	*P. pubescens*	4591	Norstictic	±	>1	0·10 (0·05–0·2)	–	+	–	±
A	*P. pubescens*	4783	Norstictic	+	>1	0·13 (0·1–0·2)	±	–	–	–
A	*P. pubescens*	5073	Norstictic	+	1–5	0·18 (0·1–0·3)	±	–	–	–
A	*P. pubescens*	5074	Absent	+	>1	0·16 (0·1–0·3)	±	–	±	–
A	*P. minuscula*	5079	Norstictic	+	<1–2	0·15 (0·1–0·2)	–	–	+	+
A	*P. minuscula*	5080	Norstictic	+	<1	0·15 (0·1–0·2)	–	+	+	+
A	*P. minuscula* (RefSpec)	4360	Absent	–	<1	0·18 (0·1–0·3)	–	+	±	+
A	*P. minuscula*	4367	Norstictic	+	<1	0·16 (0·1–0·2)	–	+	+	+
A	*P. pubescens*	4364	Absent	+	<1–1·5	0·17 (0·1–0·3)	±	+	+	+
A	*P. pubescens*	4201	Norstictic	–	<1–2	0·22 (0·2–0·3)	–	±	±	–
A	*P. pubescens*	4668	Absent	–	<1	0·15 (0·1–0·2)	–	+	+	+
A	*P. minuscula*	4590	Greyish pale spot	+	<1–1·5	0·15 (0·1–0·2)	+	+	–	±
A	*P. minuscula*	4670	Brownish spot	±	<1	0·16 (0·1–0·3)	±	+	±	+
A	*P. pubescens*	4199	Norstictic	–	<1–2	0·16 (0·1–0·3)	–	±	±	–
A	*P.* aff. *minuscula*	5071	Norstictic	+	>1	0·20 (0·1–0·3)	+	+	–	–
A	*P.* aff. *minuscula*	5072	Norstictic	+	>1	0·17 (0·1–0·3)	±	–	–	–
B	*P. pubescens*	5081	Norstictic	–	<1–1·5	0·15 (0·1–0·2)	–	+	–	±
B	*P. pubescens*	5082	*cf.* Gyrophoric	–	<1–1·5	0·17 (0·1–0·3)	perforated	–	–	–
B	*P. pubescens*	5078	*cf.* Gyrophoric	–	<1–2	0·22 (0·1–0·4)	–	–	–	–
B	*P. pubescens* (RefSpec)	4365	Absent	–	>1	0·16 (0·1–0·2)	–	–	–	–
B	*P. pubescens*	4669	Norstictic	–	>1	0·17 (0·1–0·3)	–	±	–	–
B	*P. pubescens*	4198	Norstictic	–	>1	0·17 (0·1–0·3)	+	–	–	–
B	*P. pubescens*	4196	Norstictic	–	>1	0·17 (0·1–0·3)	+	±	±	–
B	*P. pubescens*	4197	Norstictic	–	>1	0·19 (0·1–0·3)	+	±	+	±
B	*P. pubescens*	3709	Absent	–	<1–2	0·14 (0·1–0·2)	±	±	–	–
B	*P. pubescens*	4671	Norstictic	–	>1	0·17 (0·1–0·2)	±	±	–	–
B	*P. pubescens*	4200	Absent	–	>1	0·15 (0·1–0·2)	±	–	–	–
B	*P. pubescens*	4589	Absent	–	>1	0·21 (0·1–0·3)	+	±	–	–
B	*P. pubescens*	3919	Absent	–	>1	0·21 (0·1–0·3)	+	–	–	–

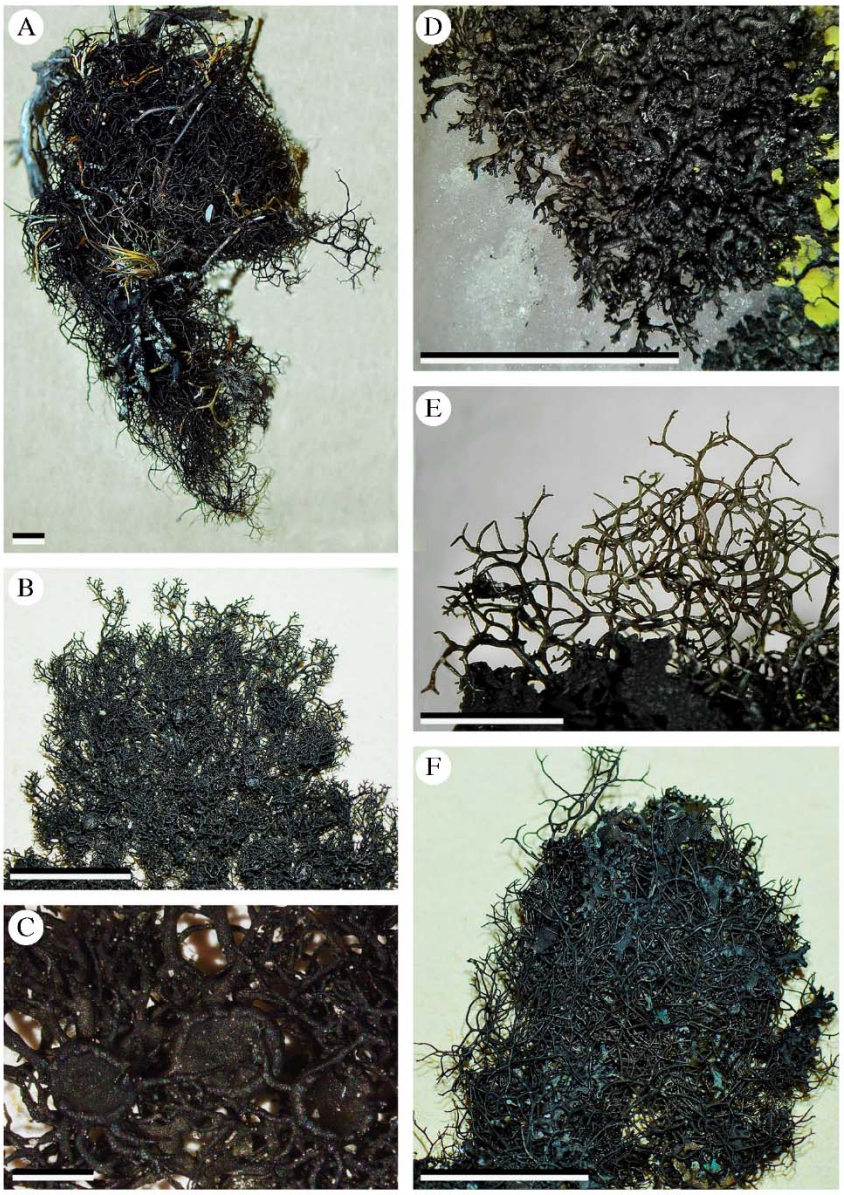

FIG. 2. Variability in *Pseudephebe* species. A–D, *P. minuscula*; A, long branched fruticose habit (NMW. C.2015.004.8); B, short to intermediate, more typical, branched habit (S F149958, sequenced reference specimen); C, specimen with apothecia in detail (S F149958, sequenced reference specimen); D, extreme subcrustose habit (hb. N. Noell 1561). E & F, *P. pubescens*; E, long branched fruticose habit (MAF-Lich. 20100); F, typical habit (S F149572, sequenced reference specimen). Scales: A, B, D–F = 5 mm; C = 1 mm.

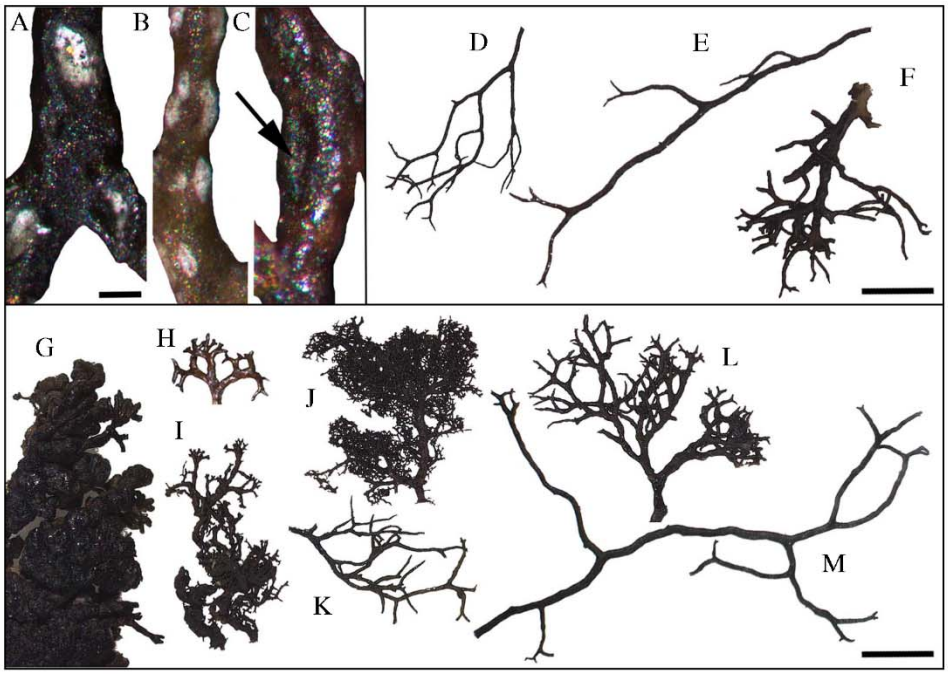

FIG. 3. Pseudocyphellae and branching variability in *Pseudephebe*. A–C, pseudocyphellae in *P. minuscula*: A, perforated (MAF-Lich. 20100); B, superficial (MAF-Lich. 19472); C, inconspicuous (MAF-Lich. 20103). D–F, *P. pubescens* branching variability; D, S F149572; E, MAF-Lich. 19474; F, MAF-Lich. 20101. G–M, *P. minuscula* branching variability; G, hb. N. Noell 1442; H, MAF-Lich. 20103; I, hb. N. Noell 1561; J, S F177970; K, MAF-Lich. 19475; L, MAF-Lich. 19472; M, NMW.C.2015.004.8. Scales: A–C = 0·1 mm; D–M = 2 mm.

TABLE 3. *Characters giving the most discrimination for the separation of* Pseudephebe *species, based on genetically analyzed material. An unequivocal identification to species is only possible using DNA barcoding.*

Character	*Pseudephebe minuscula*	*Pseudephebe pubescens*
Habit	Rarely subcrustose, small to large fruticose	Never subcrustose, small to medium fruticose
Thallus size (diameter)	Usually less than 3 cm, but reaching more than 8 cm	Usually less than 5 cm
Internode length	Usually less than 1 mm, but reaching 7 mm	Usually 1 – 3 mm, but sometimes less than 1 mm
Compressed old branches	Frequent	Rare
Flattened branching	Frequent	Rare, but never very flattened
Richly branched tips	Frequent	From absent to present in the same specimen
SSU-3' 1516 intron	Frequent	Absent

Species delimitation

Pseudephebe specimens fall into two genetically well-isolated clades (clades A and B) separated by an unexpectedly long branch (Fig. 1). The morphological characters traditionally used to separate these species were not, however, fully congruent with the two clades.

Clade B included specimens of *P. pubescens* conforming to the traditional concept of the

TABLE 4. *Species delimitation prediction according to each method and marker.*

Method/Marker	ITS	*Mcm7*	*RPB1*	Concatenated
ABGD	A, B	A, B	A, B	A, B
PTP	A, A', B	A, B	A, B	A, B
BP&P	–	–	–	A, B

A, A' and B refers to clades named in Fig.1. ABGD = Automatic Barcode Gap Discovery; PTP = Poison Tree Processes; BP&P = Bayesian Phylogenetics and Phylogeography

species (Brodo & Hawksworth 1977), but also a small number which were similar morphologically to *P. minuscula*. In contrast, 42% of the specimens from clade A were originally identified as *P. minuscula*, and the remaining 58% had been named *P. pubescens* or *Bryoria mariensis*.

Clade A (Fig. 1) contained a much greater genetic variability than clade B (here assigned to *P. pubescens*), with branch lengths and a topology similar to other alectorioid genera with multiple species (Velmala *et al.* 2014). Subclade A' (Fig. 1) is well supported and comprised specimens from western North America. That material has thin elongated terminal branches that are themselves minutely branched at the end; we initially speculated that this might merit recognition as a separate taxon, but discovered that this character was also evident in other clades.

Species delimitation approaches (Table 4) confirmed the existence of two species as the most probable scenario for the genus since no genetic gap was detected within clade A (Leavitt *et al.* 2015). We therefore assign clade A to *Pseudephebe minuscula* and clade B to *P. pubescens*. Although the two species overlap morphologically, an assay with a node dating analysis established that they could have split from a common ancestor around 9·5 to 11·6 mya (see Supplementary Material S3, available online).

Taxonomy

Where morphological identification cannot be made with confidence, species-level identification in *Pseudephebe* should be carried out by molecular methods. In cases where this is not possible, we suggest that collections should be referred to as either *P. pubescens* s. lat. or *P. pubescens* aggr., as discussed by Crespo & Lumbsch (2010). We prefer 'aggr.', as that term is used for plants, particularly in cases covering two or more named species.

In order to leave no doubt in the application of names to the two major clades distinguished here, and for assigning sequences obtained from fresh collections, we designated a sequenced reference specimen for both *Pseudephebe* species.

We have not reinvestigated species names other than those listed below, viz. *Alectoria biformis* (Vain.) Dodge, *A. congesta* (Zahlbr.) Dodge, *A. intricata* Hue, and *A. nigerrima* Hue, nor the application of numerous infraspecific names employed in the group. Information on these is provided in Hillmann (1936), Lamb (1948, 1964), Hawksworth (1972), Brodo & Hawksworth (1977), and Myllys *et al.* (2011). Many of these names are based on material from Antarctica, and are likely to represent *P. minuscula* as circumscribed here because of the morphology, but ideally they should be re-collected and sequenced from the type localities to verify their status.

Pseudephebe minuscula (Nyl. ex Arnold) Brodo & D. Hawksw.

Opera Bot. **42:** 140 (1977).—*Imbricaria lanata* var. *minuscula* Nyl. ex Arnold, *Verh. Zool.-Bot. Ges. Wien* **28:** 293 (1878).—*Parmelia minuscula* (Nyl. ex Arnold) Nyl., *Bull. Soc. Linn. Normandie, sér. 4* **1:** 205 (1887); type: Finland, Lapponia enontekiensis, Enontekio, in alpe Pietsovaara prope Kilpisjärvi, 1867, *J. P. Norrlin* (H-NYL 34355—lectotype ?, designated by Brodo & Hawksworth 1977: 141 [as "34255"] but see below); Sweden, Jämtland, Undersåker sn, Välliste, topplatån,

1300 m VSV om Norra stugan, 63°16'07"N, 13°08'16·1"E, fjällhed, klippor, på stenblock, 22 July 2009, *G. Odelvik* 9152 (S F149958—*reference specimen selected here*, DNAcode 4360; GenBank accession numbers: ITS KU647304, *RPB1* KU668527, *Mcm7* KU668490).

Alectoria pacifica Stizenb., *Proc. Calif. Acad. Sci., ser. 2* 5(2): 537 (1895): type: [Mexico], Insulae californica Guadalupe, supra terram humosam, 1875, *E. Palmer* (ZT Myc55359—holotype; US—isotype n. v.).

Bryoria mariensis Øvstedal *et al.*, *Lichenologist* 44: 487 (2012); type: Falkland Islands, West Falkland, Port Howard, on summit of Mt. María, UTM 21F UC 2079, 2158 ft [658 m], feldmark, 28 January 1968, *H. A. Imshaug* 41327 *&* *R. C. Harris* (MSC 80870—holotype).

(Figs 2A–D & 3A–C, G–M)

Myllys *et al.* (2011: 140) designated a different specimen from that selected by Brodo & Hawksworth (1977: 141) as lectotype for Nylander's "*minuscula*". They cited an undated specimen from Austria collected by Arnold (H-NYL 34356), without discussion, but presumably as they considered that a lectotype had to be chosen only from material studied by the validating author, in preference to one from Finland collected in 1867 and studied by Nylander (H-NYL 34355), which was selected by Brodo & Hawksworth. This case is, however, complex as Arnold (1878: 293) also referred to earlier usages of the name by Nylander, Stizenberger, and himself. There is no indication that Arnold was intending to do anything but cite an identification in a floristic list of species in a region of Austria. Under Art. 9.3, original material in validating descriptions can include materials not seen by the validating author. Also mentioned by Arnold is "Nyl. Lapp. Or. 120", which is a direct reference to Nylander material, and it seems preferable to have a Finnish specimen as lectotype as Arnold cited Nylander as the author. "Lapp. Or. 120" would seem to be a cryptic reference to an exsiccate of Fellman issued in 1865 which, however, has "*Parmelia lanata* f. *minuscula*" as no. 83, while no. 120 is "*Lecanora dicksonii*" (Lynge 1915–16). However, there is a Fellman specimen in H-NYL from "Karelia pomorica orientalis, Suma" dated 1863 (H-NYL 34357) and examined by DLH in 1968 which could therefore be a better candidate. The issue merits further study and a resolution of this typification is beyond the scope of the present work. Rather than complicate matters further here, we have indicated a reference specimen (RefSpec) to serve as a proxy sequenced type, following the proposal of Ariyawansa *et al.* (2014), as an interim solution to fixing the molecular application of the epithet *minuscula* in our sense.

The name *Alectoria antarctica* Dodge & Baker is excluded here as it was based on a specimen of *Pseudephebe minuscula* infected by a lichenicolous fungus. The lichenicolous fungal element was selected as lectotype for the name by Hawksworth & Iturriaga (2006: 202), who transferred it into *Carbonea*.

Specimens referred to *Pseudephebe pubescens* f. *subciliata* (Nyl.) D. Hawksw. forming appressed rosettes and with short internodes appear to belong here. They were retained under *P. pubescens* by Hawksworth (1972) as the lobes were terete rather than dorsoventrally compressed, but from the data presented here that character does not appear to be taxonomically informative and environmentally induced. Of the total samples analyzed molecularly, specimens from Austria, Chile, the Falkland Islands (Islas Malvinas), Norway, Portugal, Romania, Spain, Sweden, and the USA were clustered in the *Pseudephebe minuscula* clade.

Additional specimens examined (DNA, morphology, and chemistry examined). **Austria:** *Tirol*: Otzaler Alpen, SE of Martin Busch Haus, 46°47'57"N, 10°52'47"E, 2580 m, on gneissic boulder, 2011, *R. Türk* 49630 [Obermayer, *Lichenoth. Graec.* No. 379] (MAF-Lich. 17091, as *Pseudephebe pubescens*, DNAcode 4201).—**Chile:** *Magallanes y Antártida Chilena* (*XII Región*): Navarino Island, Cabo de Hornos Commune, Bandera Hill, 15 iii 2012, *C. Laguna Defior* (MAF-Lich. 20105, DNAcode 5073, TLC: norstictic acid; and MAF-Lich. 20106 (DNAcode 5074, TLC: no substances detected), 20110).—**Falkland Islands (Islas Malvinas):** *West Falkland* (*Gran Malvina*): Port Howard (Puerto Mitre), Mt. María, 51·60768°S, 59·58654°W, 580 m, on sandy soil in feldmark, 2015, *A. Orange* 22484 (NMW s. n., as *Bryoria mariensis*, DNAcode 5077); Mt María summit, *D. Crabtree* [*A. Fryday* 10925] (MSC, as *Bryoria* sp., DNAcode 5075).—**Norway:** *Sogn og Fjordane*: Sogn, *c.* Luster, 61°31'58"N, 07°50'49"E, 1316 m, on rocky soil with moss and lichens, 12 viii 2015, *C. G. Boluda & N. Calpena* (MAF-Lich. 20101, DNAcode 5082, TLC: *cf.* gyrophoric acid). *Nordaland*: E6 road, contact with the

Arctic Circle, 66°34'36"N, 15°20'57"E, 679 m, on soil with mosses and lichens, 14 viii 2015, *C. G. Boluda & N. Calpena* (MAF-Lich. 20107, DNAcode 5079, TLC: norstictic acid).—**Portugal:** *Beira Baixa*: Covilha, Serra da Estrela, Torre, 40°19'N, 07°36'W, 1966 m, on granitic boulders, 11 vi 2014, *V. J. Rico* (MAF-Lich. 19472, DNAcode 4590). *Minho*: Peneda-Gerés Natural Park, *c.* Castro Laboreiro, 42°01'N, 08°08'W, 1028 m, 9 ix 2014, *C. G. Boluda & V. J. Rico* (MAF-Lich. 19473, DNAcode 4670).—**Romania:** *Hunedoara*: Vrdele Pass, 45°20'40"N, 23°39'31"E, 2115 m, 12 x 2014, on siliceous rock, *C. G. Boluda & E. Araujo* (MAF-Lich. 19475, as *P. pubescens*, DNAcode 4668).— **Spain:** *Asturias*: Caso, Campo de Caso, Redes Natural Park, Valdebezón, from Monasterio River to peña'l Vientu, 43°05′21′′N, 05°19′33′′ W, 1553 m, S slope, on quartzite, 2012, *V. J. Rico* 4423 (MAF-Lich. 17838, as *P. pubescens*, DNAcode 4199). *Segovia*: La Granja de San Ildefonso, *c.* Puerto de Navacerrada to Puerto de Cotos road, brook of Los Puentes, towards the Loma del Noruego, 40°47'36"N, 03°57'12"W, 1900 m, on granite, 2014, *V. J. Rico* 4614 (MAF-Lich. 20103, DNAcode 5071, TLC: norstictic acid; MAF-Lich. 20104, DNAcode 5072, TLC: norstictic acid).—**Sweden:** *Jämtland*: Kall par., Skäckerfjällen Nature Reserve, Rutsdalen, SW slope of Dörsvalen, 63·80775°N, 12·84044°E, 700 m, on siliceous rock in boulder area, 2010, *M. Westberg* 10-078 (S F177970, DNAcode 4367). *Lycksele Lappmark*: Tärna sn, Atoklintens, 65°40·904'N, 14°38·310'E, on rock, 2012, *G. Odelvik* 12609, *M. Hamnede & L. Hedenäs* (S F240229, DNAcode 4364).—**USA:** *Alaska*: Fairbanks North Star Co., Steese Highway, Eagle Summit, 65·4867, −145·4153, 1100 m, alpine tundra, on rocks, 2011, *R. Rosentreter* 17334, 17292 *et al.* (SRP L8791, L8806, as *P. pubescens*, DNAcode 4339, 4332). *California*: Placer Co., Tahoe Rim Trail just north of Blockway Summit Trailhead, 39·2646°N, 120·0607°W, 2361 m, on granite, 2015, *N. Noell* 1581 *& J. Hollinger* (hb. N. Noell, as *P. pubescens*, DNAcode 4781). *Montana*: Deerlodge Co., Beaverhead-Deerlodge National Forest, *c.* Four Mile Basin, 46°05·680'N, 113°13·918'W, 2438 m, on granite boulders, 2009, *St. Clair* 16685 [St. Clair *et al.* Lich. W. N. Amer. no. 145] (S F175892, as *P. pubescens*, DNAcode 4363); Jefferson Co., just E of Homestake Pass on I90, 45°55'01"N, 112°22'33"W, 6200 ft [1890 m], on boulders, 2006, *C. M. Wetmore* 94730 (S F144171, as *P. pubescens*, DNAcode 4362). *Nevada*: White Pine Co., Great Basin National Park, Mount Wheeler, 38·9859°N, 114·3148°W, 3981 m, rocky summit, on quartzite, 2014, *N. Noell* (1442) *& J. Hollinger* (hb. N. Noell, DNAcode 4784). *Washington*: Spokane Co., Turnbull National Wildlife Refuge, Kepple Lake overlook, 47·441°N, 117·528°W, 700 m, on top of basalt on forest floor, 2015, *N. Noell* 1557, *J. Allen & R. O'Quinn* (hb. N. Noell, as *P. pubescens*, DNAcode 4783).

Pseudephebe pubescens (L.) M. Choisy

Icon. Lich. Univ., ser. 2 **1:** *sine pag.* (1930).—*Lichen pubescens* L., *Sp. Pl.* **2:** 1155 (1753); type: Dillenius, *Hist. Musc.*: pl. 13, fig. 9 (1742) (lectotype designated by Jørgensen *et al.* 1994: 343); *sine loc.* (LINN 1273.286—epitype designated by Jørgensen *et al.* 1994: 343); Sweden, Jämtland, Undersåker sn, Välliste, topplatån, 1300 m VSV om Norra stugan, 63°16'07"N, 13°08'16·1"E, fjällhed, klippor, på stenblock, 22 July 2009, *G. Oldevik* 9115 (S F149572—*reference specimen selected here*, DNAcode 4365; GenBank accession numbers: ITS KU647317, *RPB1* KU668513, *Mcm7* KU668477).

(Figs 2E–F & 3D–F)

Jørgensen *et al.* (1994: 343) pointed out that the specimen in the Linnean collections under the name *Lichen pubescens* (LINN 1273.286), which had been cited as lectotype by Hawksworth (1972: 235), was annotated in the handwriting of Linnaeus' son and not Linnaeus himself, something also pointed out by Howe (1912: 201). In the absence of evidence that the specimen was part of the original material studied by Linnaeus prior to publication of the name, it is not eligible as a lectotype and the Dillenian plate has to be used. Jørgensen *et al.* (1994: 343) consequently designated LINN 1273.286 as epitype. As there appears to be no mechanism to change an epitype once selected, without going through the formal conservation process, we have indicated a reference specimen (RefSpec) to serve as a proxy sequenced type to fix our application of the epithet *pubescens*, as for *minuscula* (see above).

We selected a sequenced reference specimen from northern Sweden, as Linnaeus (1753: 1155) gave the habitat information as "Habitat in Europa septentrionali, Lapponia, Suecia". Dillenius (1742: pl. 13, fig. 9) gives the locality as "In rupibus Groenlandie leóta & ab me delata fuit a Franc. Soldano, Chirurgo." (loc. cit.: 66), so from Greenland where it was evidently collected by Francesco (or perhaps Franco) Soldano (fl. 1700–1740), a surgeon and friend of Dillenius known to have collected at several sites in Greenland as far as 81°N. Some specimens of Soldano are evidently still present in the Sherard Herbarium in OXF (Clokie 1964). Specimens in that herbarium are not available for loan and have not been studied by us. Although potentially of historical interest, they are not now pertinent to the typification of the Linnean name as an epitype has already been designated by Jørgensen *et al.* (1994).

Of the samples analyzed molecularly, specimens from Norway, Spain, Sweden, and regions of Switzerland clustered in the *Pseudephebe pubescens* clade.

Additional specimens examined (DNA, morphology, and chemistry examined). **Norway:** *Sogn og Fjordane*: Sogn, *c.* Luster, 61°31'58"N, 07°50'49"E, 1316 m, on rocky soil with mosses and lichens, 12 viii 2015, *C. G. Boluda & N. Calpena* (MAF-Lich. 20100, 20102, DNAcode 5081, 5080, TLC: norstictic acid). *Nordaland*: E6 road, contact with the Arctic Circle, 66°34'36"N, 15°20'57"E, 679 m, on soil with mosses and lichens, 14 viii 2015, *C. G. Boluda & N. Calpena* (MAF-Lich. 20108, DNAcode 5078, TLC: *cf.* gyrophoric acid).—**Spain:** *Asturias*: Caso, Campo de Caso, Redes Natural Park, surroundings of Ubales Lake, N slope of Cascayón pike, 43°06'05"N, 05°21'16"W, 1750 m, on quartzite, quartzite vertical wall, on sedges, other lichens and bryophytes, 2012, *V. J. Rico* 4490, 4498, 4513 (MAF-Lich. 17907, 17915, 17930, DNAcode 4198 (TLC: norstictic acid), 4196, 4197); 43°06'09"N, 05°21'11"W, 1750 m, on quartzite, 2012, *C. G. Boluda* 88 (MAF-Lich. 18112, DNAcode 3709). *León*: Riaño, Picos de Europa, close to the village, 43°07'47"N, 04°58'59"W, on siliceous conglomerates, 1 xi 2014, *C. G. Boluda & N. Calpena* (MAF-Lich. 19474, DNAcode 4671). *Teruel*: Orihuela del Tremedal, Nuestra Señora del Tremedal Sanctuary, 30TXK143874, 1725 m, *Pinus sylvestris* forest, on quartzite, 2010, *V. J. Rico* 4032 & *M. Vivas* (MAF-Lich. 16841, DNAcode 4200). *Zamora*: Sanabria Lake, close to the Laguna de los Peces, 42°09'50"N, 06°44'10"W, 1641 m, rocky areas among bushes, 28 vii 2013, *C. G. Boluda & N. Calpena* (MAF-Lich. 19470, 19471, DNAcode 4589, 3919).—**Switzerland:** *Uri*: *c.* Wassen, 46°45'08"N, 08°29'01"E, 2210 m, alpine siliceous rocky outcrops, 15 vi 2014, *C. G. Boluda & Ch. Scheidegger* (MAF-Lich. 19476, DNAcode 4669).

Discussion

Pseudephebe is confirmed as a separate genus in the alectorioid clade of *Parmeliaceae*, with two genetically isolated clades, recognized here as *P. minuscula* and *P. pubescens*. Pseudocyphellae (Fig. 3), norstictic acid, and other extrolites are common characters in the genus, although these have not previously been recognized. *Pseudephebe pubescens* and *P. minuscula* are not always morphologically distinguishable, as indicated by annotations of specimens examined by numerous lichenologists, and our molecular data. The morphological variability of *P. pubescens* overlaps with that of the more variable *P. minuscula*. Many specimens are distinguishable with confidence only by DNA analyses, especially of the ITS region, thus proving it to be cryptic. Nevertheless, specimens with extreme morphologies (subcrustose thalli, considerably flattened branching patterns or very compressed branches), as well as samples with an intron at the end of the SSU-3' locus, are most likely to belong to *P. minuscula*.

In some cases, as in several Norwegian samples analyzed (MAF-Lich. 20108, 20107, 20102, 20100 and 20101), *Pseudephebe minuscula* and *P. pubescens* grow together with comparable morphology, suggesting that the final thallus form is environmentally determined, particularly in *P. minuscula*. Comparable situations in which thallus morphology appears to be related to environmental factors have been described in a range of other lichens (Hawksworth 1973) but many of those examples have yet to be examined by molecular methods. The underlying mechanisms of this process are unknown, and perhaps could be elucidated by gene-expression studies in due course. The possibility that some extrachromosomal "evo-devo" processes (Sultan 2015) are involved may also merit investigation when technically feasible.

SSU-3' 1516 introns are common in *Parmeliaceae* (Gutiérrez *et al.* 2007), but a variable presence was not previously known to occur within a single species, as specimens with and without an intron generally belong to different cryptic species (Molina *et al.* 2011*a*, *b*). In other lichen groups such as in *Physcia*, belonging to *Physciaceae* (*Caliciales*), similar introns in close-by regions (e.g. the LSU) are common and variable in a single species and even in the same specimen (Simon *et al.* 2005). PCR is a process in which the commonest or the most competitive locus allele can be fixed on the resulting sequence, masking the probability of detecting within-locus variability. Two of our samples were found to have intron presence and absence. As the SSU is a multi-copy locus, we cannot establish if this variability is among different regions of the branches, different fungal partners growing in a single thallus or inside a single nucleus. This could indicate that SSU-3' 1516 intron is an unstable element that can be deleted

from the genome and regained thorough 'homing' (homing endonuclease) or reverse splicing mechanisms (Haugen *et al.* 2004; Bhattacharya *et al.* 2005; Reeb *et al.* 2007). This intron seems present as an ancestral character in clade A and then secondarily lost independently in some clades, individuals, thallus regions, or even in some of the copies in a single fungal nucleus.

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Supplementary Material

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0024282916000426. This includes: 1) a list of additional specimens studied morphologically and chemically, and arranged according to the original identifications; 2) data on apothecial morphological characters in *Pseudephebe* specimens studied; and 3) an assay analysis to node ages estimation.

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